

**THE MASS PRODUCTION OF CONIFER TREE HYBRIDS  
PHASE I. DEVELOPMENT OF CELL SUSPENSION –  
EMBRYOID – PLANTLET TECHNIQUE**

**Project 3223**

**Report Five  
A Progress Report  
to**

**MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY**

**August 1, 1977**

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

THE MASS PRODUCTION OF CONIFER TREE HYBRIDS  
PHASE I. DEVELOPMENT OF CELL SUSPENSION -  
EMBRYOID - PLANTLET TECHNIQUE

Project 3223

Report Five

A Progress Report

to

MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY

August 1, 1977

# TABLE OF CONTENTS

	Page
SUMMARY	1
INTRODUCTION	7
WORKING HYPOTHESES	12
Initiation-Development Hypothesis	12
Carbon Utilization Hypothesis	13
Peroxide Regulation Hypothesis	15
IAA Oxidase Hypothesis	16
GROWTH AND ORGANIZATION IN CALLUS CULTURES	17
New Needle Callus from Douglas-fir and Loblolly Pine	17
Shoots from Needle Callus of Douglas-fir	22
Growth Medium for Loblolly Pine Callus	26
Enzyme Investigations	29
Peroxide Analyses	54
Elemental (Metal) Analyses	57
GROWTH AND ORGANIZATION IN SUSPENSION CULTURES	60
Effects of Chemical and Environmental Factors	60
Sterilization	65
Cell Cluster Size	68
Preparation of Inocula	73
Embryoids from Douglas-fir Suspensions	76
Ultrastructure of Douglas-fir Embryoids	80
Feedback Research	87
STUDENT RESEARCH	97
NEW STUDIES	100
Phenolics	100
IAA Analysis	103

Polyamines	103
Optimization of Liquid Growth Medium for Loblolly Pine Suspension Cultures	104
Washing of Suspension Inocula	107
Cell Separation by Hormones or Enzymes	109
Isolation and Growth of Embryoids	110
FUTURE PLANS	114
ACKNOWLEDGMENTS	116
LITERATURE CITED	117
RELATED PUBLICATIONS	119
GLOSSARY	120
APPENDIX	125

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

THE MASS PRODUCTION OF CONIFER TREE HYBRIDS  
PHASE I. DEVELOPMENT OF CELL SUSPENSION -  
EMBRYOID - PLANTLET TECHNIQUE

SUMMARY

Funded Project 3223 has the overall objective of the development of a reliable, low-cost method for the mass production of conifer hybrids and selected "elite" trees. The basic approach is the development of a procedure that starts with cells in suspension that can be manipulated to form embryoids, embryos, and finally plantlets and trees. Research presently emphasizes the tissue culture propagation of Douglas-fir and loblolly pine. The overall research program has been divided into studies dealing with callus (small groups of randomly dividing and unorganized cells) growing on a solid growth media and studies involving single cells and small clusters of cells growing in liquid media.

Earlier, research at the Institute (and at other institutions) resulted in the production of shoots from callus derived from parts of seeds and very small seedlings. In some instances the shoots have rooted and plantlets resulted. Procedures for growing Douglas-fir callus, and more recently loblolly pine callus, are now worked out and callus production is routine. Callus and shoots obtained from different sources of callus are being used primarily for biochemical feed-back investigations. Callus obtained from needles of Douglas-fir and loblolly pine appear to have several advantages as a starting point for investigations on the formation of shoots and/or roots (organogenesis) and embryos (embryogenesis). During this past year, a routine, reliable procedure was developed for producing large amounts of needle callus. This procedure is described in some detail in an

early section of this report. Of considerable interest, although not presently one of our major objectives, was the production of additional shoots from sub-cultured Douglas-fir needle callus. The shoots were used in enzyme and other biochemical studies for comparing organized (shoots) and unorganized growth (callus). Shoots and the shoot-producing callus are expected to contribute to our understanding of the trigger mechanism involved in shoot initiation.

A major and very important aspect of the biochemical research carried out this past year consisted of a series of studies to determine the degree of correlation between biochemical composition, enzyme activity and morphogenesis (development of shoots, roots, and/or embryos). Two main hypotheses now guide our work. These are: (1) the peroxide regulation hypothesis, which deals with the importance of maintaining proper cellular peroxide levels, and (2) the closely related IAA oxidase hypothesis, which deals with the factors that regulate levels of indoleacetic acid, an important natural auxin (plant hormone).

Comparative biochemical analyses of organized and unorganized tissue (shoots vs. callus) revealed compositional differences of possible relevance to developmental processes. Enzyme studies included the influence of plant hormones 2,4-D and BAP\* on the activity of such growth-related enzymes as peroxidase, catalase, and superoxide dismutase in Douglas-fir callus. The results indicate, for example, that hormones like 2,4-D and BAP should have an indirect long-term effect on peroxidase activity and a rapid, short-term effect on superoxide dismutase activity. Investigations involving enzyme activity/radioactive tracer techniques demonstrated the importance of isoperoxidases and indoleacetic acid oxidase activities in embryogenesis. Indoleacetic acid oxidase promoters were

---

\*See list of abbreviations in appendix.

found to promote embryoid formation in the suspension cultures. In all instances investigated, the addition of hydrogen peroxide was required to obtain IAA oxidase activity.

Growth of suspension cultures is an important aspect of the overall program. A major phase of the ongoing research program during this past year has been a series of studies that have the objective of developing standard operating procedures and near optimum growing conditions for cell suspension. Considered were media sterilization techniques, cell cluster size and a series of other chemical and environmental factors including sucrose levels, types of experimental vessels, drum speed and light quality, quantity and duration. Appropriate autoclaving and filtering procedures were developed and sucrose levels of 3.5% were found to be satisfactory. For feedback research, drum speeds of 10-15 rpm, light intensity of 4,000 lx and a 16-hour day alternating with 8 hours of darkness appears to be the best. Lower light intensity of 1,000 lx improved embryoid development. Studies dealing with cell cluster size demonstrated that callus cells can be separated and embryoid initiation improved by sieving and isolating single cells and small clusters of cells from the apparent inhibiting influence of the large clusters.

Another very important aspect of the program is the initiation of embryoids. Embryoids (small groups of cells exhibiting nonrandom cell division) have been repeatedly initiated from suspensions of small cell clusters and a few treatments produced embryoid initiation frequencies of up to 50-60%. The Douglas-fir embryoids differ in appearance and method of origin from those reported by workers studying embryogenesis in herbaceous species. Douglas-fir embryoids have enlarged to the 100-200 cell stage but have not reached the commonly observed globular, heart or torpedo embryo stage described for herbaceous species. We

presently still feel the structures we have observed, because of their early vigor and the nonrandom sequence of cell division that occurs, are embryonic. Observations made by Electron Microscopy Department on embryoids exhibiting arrested development indicate there was a well-developed mother-cell envelope which encased the cells and that the arrested development appeared to be related to a phenolic buildup within the cell vacuoles. These observations are in agreement with preliminary chemical determinations and similar situations reported in the literature, and are the basis for a new series of studies aimed at providing the cells first with a growth medium that stimulates the initiation of embryoids and then a second medium (developer medium) that prevents phenolic buildup and permits the embryoids to develop into true embryos.

Feedback research is perhaps the most interesting and exciting phase of the entire research program because this is the testing ground where insights from biochemical investigations are utilized to induce or control growth and embryogenesis. Investigations during the past year include the use of uridine diphosphate (UDP) and related additives, reduced levels of iron, the use of malate, succinate and malonate as growth regulatory agents, and the use of several chemicals that are IAA oxidase inhibitors or promoters. UDP quite consistently increased the production of embryoids with one of the more successful treatment sequences being to treat with UDP for three days and then transfer the cells into a medium with pyrophosphate and a pyrophosphatase inhibitor. Reducing iron levels from 5.6 to 1.4 mg/liter improved greening and, to a lesser extent, embryogenesis.

The feedback results that are presently most exciting are the results mentioned earlier under enzymes where it was pointed out that IAA oxidase promoters, such as p-coumaric acid, induce embryoid formation and the IAA oxidase inhibitors reduce the initiation of embryoids. Equally interesting is the fact



that embryoid formation has also been observed when hydrogen peroxide is added, thus confirming the connection between IAA oxidase and the peroxidase regulation hypotheses.

Student research has become an increasingly important phase of the overall program. Five students are either working on, or have completed, studies that relate to the tissue culture program. Study topics vary considerably and include a thesis under way on a growth inhibitor, methylglyoxal, which has the potential of reducing growth and stimulating morphogenesis and two special studies with topics on the production, isolation, fusion and culture of sterile protoplasts (cells in which the cell wall was removed).

Research by post-doctoral fellows is also an important aspect of the program. Dr. Kimani Waithaka is responsible for the work underway on the "Development of a Growth Medium for Loblolly Pine Suspensions" and Dr. Francis Hsu has recently started the work described under new studies on the "Analysis of Levels of IAA in Various Types of Tissue."

The specific plans and objectives are spelled out in detail at the end of each major section of the report. Research will be continued in certain areas and new studies are planned that will allow us to: (1) Select tissues that are designated by industry to permit positive genetic gains; (2) Determine the site or origin of embryoid initiation (callus or suspension cultures), isolate embryoids and stimulate their development into true embryos; (3) Obtain microscopic evidence of the organizational structure of developing embryoids, including physical connections between cells and the development of cells having special functions; (4) Obtain evidence concerning the importance of maintaining proper cellular peroxide levels (peroxide regulation hypothesis) in the initiation and enlargement of embryoids;

(5) Develop a near optimum loblolly pine liquid growth media, improve existing biochemical feedback assay methods and use this information in embryoid initiation studies; (6) Develop methods for preventing phenolic buildups which appear specific to conifer tissue and are believed to be inhibiting embryoid enlargement; (7) Evaluate levels of IAA and other growth hormones in tissue from various treatments and origins as part of the research on tested morphogenetic systems and the IAA oxidase hypothesis; (8) Investigate factors involved in the preliminary scale-up of cell numbers so as to provide for future controlled morphogenesis investigations.

## INTRODUCTION

Numerous questions and comments during the past year regarding the Institute's Tissue Culture Program prompted the discussion that follows concerning the objectives, philosophy and the research approach being employed. The long-range objective of our research program is the development of a procedure for the mass production of selected "elite" trees and conifer hybrids. The selected "elite" trees are expected to be appropriately evaluated trees from company lands. It is anticipated that the hybrids to be propagated will be produced by either vegetatively propagating cultured cells from existing promising sexual (conventionally produced) hybrids or by propagating the products of fused or transformed protoplasts.

We, as well as other workers, have produced shoots directly from different parts of conifer seeds and germinating seedlings, and have produced shoots from callus initiated from needles of seedlings 1-2 years old. However, we have not yet propagated tissues from older trees that have been proven to be genetically superior. Nor have we fused naked cells (protoplasts) or propagated trees from unfused protoplasts. Currently, we are attempting to characterize the trigger mechanism of cell differentiation and embryogenesis in liquid suspension cultures of Douglas-fir. This is a cooperative team effort among the tissue culture, biochemical and electron microscope laboratories, and presently involves seven professional, four technical, and five student workers.

The goal of the professional staff is to propagate trees from cultured tree cells, but four students are working on various aspects of the production, isolation, culture, wall regrowth, and fusion of protoplasts. We hope that by the time we can propagate trees from needle cells of older trees, the student

research will have solved the basic problems associated with protoplast culture, and we can combine the two methods and propagate trees from protoplasts.

Most tree tissue culturists in other laboratories are developing methods of propagating conifer trees from parts of seeds or seedlings. This approach is appropriate for the evaluation of racial and family differences important to tree-improvement programs. However, since our objectives are different, we feel we cannot solve problems of propagating older trees or fusion products except by using experimental material closely approximating these tissues. In addition, trees grown from seed or seedling parts will not be genetically identical to either parent, but rather will be some mixture of the genetic makeup of both parents. Our long-range objective demands that we reproduce the exact genetic composition of rigorously selected "elite" trees and/or cells of hybrid origin.

Biochemical investigations into enzyme activity demonstrated that enzymatic systems in seed (cotyledon) callus may differ from those in stem or needle callus. These enzyme systems apparently confer a special capacity to the developing seedlings that is not found at any other time during the life cycle. This may explain why shoot initiation has been relatively easy from seed and seed callus, but difficult from callus initiated from parts of older trees. Shoot-producing seed callus has been used for comparative biochemical analyses, in attempts to determine the trigger mechanism of shoot initiation (organogenesis). However, for our purposes, needle callus has been selected as a primary source of cells for cell suspension propagation studies. Needle callus was selected because preliminary investigations demonstrated that the enzyme systems are apparently compatible with morphogenesis, a condition that does not occur in stem callus.

Moving to the next question, concerning the type of culture system to use, we have a choice of initiating shoots from callus growing on the solidified surface of nutrient medium, or producing plantlets and trees from single cells or cell aggregates in liquid suspension cultures.

Although we and others have produced shoots from callus initiated from needles of seedlings 1-2 years old, under the best conditions the trees propagated from callus tissue will only number in the hundreds or at best in the thousands. Our goal is to develop an effective, efficient and relatively cheap method of producing thousands or hundreds of thousands of trees from cells growing in a few flasks. We believe that the commercial mass production of trees must deal with high numbers, and that the development of appropriate methods could have an enormous impact on reforestation, forest economics, ecology and on the future survival of industries dependent on an abundant supply of cellulose. For these reasons we think that the most feasible method of mass production is embryogenesis from single cells or cell aggregates in suspension cultures.

Figure 1 shows the basic difference between propagation from callus and from suspensions. Morphogenesis is a general term indicating the development of structures from unorganized tissue. Here, morphogenesis is separated into two types of development: organogenesis is the formation of organs (roots or shoots) from preformed clusters of cells (meristemoid) in callus tissue, and embryogenesis is the bipolar division and development of single cells into embryoids\* and embryos similar to stages of normal seed-embryo development following fertilization in seed plants. In organogenesis, either a root or a

\*The term embryoid conveys no commitment to further development. Rather it is a term of convenience designating a group of cells with nonrandom orientation that resembles the early stages of normal seed embryogenesis. On the other hand, the term embryo implies the ability to grow into a plant.

## MORPHOGENESIS

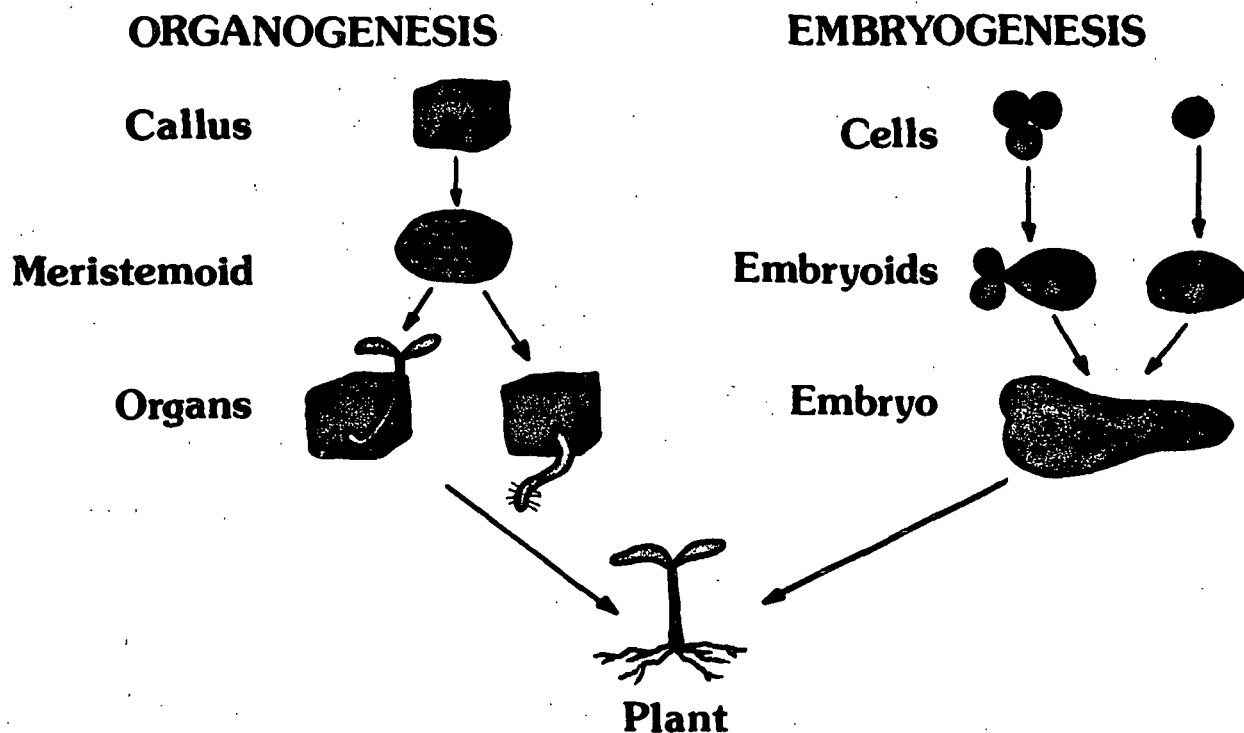


Figure 1. Illustrated and Defined Are the Two Terms Organogenesis and Embryogenesis that Are Commonly Recognized When the More General Term Morphogenesis Is Used

shoot will grow from each meristemoid, but not both. In a few cases, shoots growing from callus have also grown roots while still attached to the callus, but generally the shoots must be excised and rooted separately in order to obtain a complete plant. However, in embryogenesis, each embryo will grow both a root and a shoot from opposite ends, and thus form a complete plant directly from the one structure. The significance of this difference is that meristemoids are formed from existing cells in callus and growth is accompanied by an increase in RNA, starch and protein. The location of each meristemoid within the callus is controlled by the numerous chemical and environmental gradients in the callus, resulting from the interaction among many cells in

contact with each other. On the other hand, embryoids are initiated from single cells floating free or from small cell clusters, and apparently this process is inhibited by large callus masses. Embryogenesis thus becomes more difficult to control because most of the factors necessary to initiate embryoids must come from the medium made by the investigator, and probably few factors come directly from other cells. However, if embryogenesis can be worked out for one species of conifer, it is generally believed that very similar critical biochemical conditions will trigger embryogenesis in other conifer species.

Our basic approach has been for the tissue culture lab to continue to improve the basic medium and environmental factors empirically for suspension cultures, and the biochemical lab to initiate feedback experiments based on metabolic and enzymatic studies. Treatments used in feedback studies are mostly chemical additives, and the best ones are incorporated into our basic methods. Several good additives have been tried that have increased the frequency of embryoids in suspensions, relative to untreated controls. We have repeatedly started with suspensions with few or no differentiated cells, and after 1-2 weeks have observed significant numbers of embryoids. Small embryoids of 3-5 cells have been initiated in frequencies of over 50%. Fewer embryoids have been produced of up to 15-30 cells, and rarely large embryoids of about 200 cells have resulted. Our problem to date has been that we could produce embryoids, but they have not yet enlarged and developed into plants.

The complex biochemical nature of the problem of controlling cell growth from a single cell through to a plantlet has resulted in the development of several working hypotheses that are interrelated and are believed to have an important role in the control of morphogenesis. These hypotheses are discussed in some detail in the section that follows, and serve as an important starting point in the search for the trigger mechanism controlling embryogenesis.

## WORKING HYPOTHESES

The very complex nature of the biochemistry of morphogenesis (organogenesis and embryogenesis) in plant tissue culture systems has made it necessary to develop several working hypotheses which allow us to isolate and examine in considerable detail certain aspects of the overall morphogenesis problem. Four working hypotheses have been developed that are believed to be important and must be properly understood in order to trigger embryogenesis. It is also important to note that the hypotheses are not mutually exclusive and it may well turn out that each is important in its own way in making the system go. These hypotheses will be referred to in various sections of the report that follow and so, to make these latter comments more meaningful, the hypotheses involved are briefly defined and discussed in the paragraphs that follow.

### INITIATION-DEVELOPMENT HYPOTHESIS

Based upon observations here and in other laboratories (1), it appears that it may be useful to separate adventive embryogenesis into at least two stages, namely, initiation (induction) and development. In essence this hypothesis is that some factors effective for initiating embryoids are not necessarily desirable for the further development of embryoids. Operationally, this means that, as soon as embryoids are formed through the use of inducing agents, they should be separated from such agents and transferred to a medium designed for development.

There are two major observations that have prompted this hypothesis:

(1) accumulation of apparently toxic phenolics in embryoid cultures as well as other conifer cultures, and (2) induction of embryoids under oxidizing conditions. For example, if the IAA\* oxidase hypothesis is correct, it may be essential to lower

---

\*See list of abbreviations in appendix.



the IAA concentration temporarily by oxidation to induce organization, but prolonged contact with oxidants like peroxide may inhibit proliferation of the organized state. Such inhibition may be direct, e.g., via oxidation of functional groups on proteins, or, indirect, through accumulation of, e.g., oxidized and condensed phenolics.

Our embryoids begin to accumulate brown material within a week or two after they are initiated, and they soon die, particularly if the medium is not changed once or twice a week. Other workers, notably in coffee tissue culture (2), have found that the accumulation of oxidized phenolics inhibited growth and development of cultured tissue. Conifers have a well-developed phenolic system which could be the main source of inhibitors impeding morphogenesis both in callus and in cells in suspension. Perhaps this inhibition can be overcome in callus by the addition of cytokinin, but we and others know that cytokinin inhibits both growth and embryogenesis in suspension cultures. Therefore, if this is a serious problem, another way is needed to neutralize the phenolic inhibition in our liquid cultures. We are testing those factors that have reduced phenolic production in other plant species, and we have some indication that a few might be applicable to conifers. Some factors that stimulate embryoid formation may inhibit further development into embryos because they also affect the metabolism of phenolics. All of our embryoids in the past have died or failed to enlarge beyond a certain point. We are optimistic that embryoids placed in a developer medium will enlarge and form embryos and plantlets. The use of the developer medium to condition suspension cells prior to initiation may also be useful.

#### CARBON UTILIZATION HYPOTHESIS

It is widely observed that sucrose is the best carbon source for plant tissue culture despite the fact that it must be cleaved to yield glucose if it is

to be utilized. If sucrose is split hydrolytically, ATP will be needed to bring the resulting glucose into metabolism as would be the case if glucose itself were fed as the carbon source. We have evidence that callus tissues contain enzymes that would allow an alternate pathway to glucose-6-phosphate via UDP glucose (Fig. 2). This alternate route would not require the high energy compound, ATP, but would require UDP and pyrophosphate. Because of enzyme localization and other considerations, one might be more successful delivering exogenous UDP than ATP to such an *in vivo* system. In order for UDP addition to be effective, pyrophosphate may also have to be added along with some stabilizing factor to prevent its unproductive breakdown as indicated. Although the various metabolites shown here have other possible metabolic fates, only the reactions of immediate interest are shown. In essence, this hypothesis is that the callus or suspension cells may be unable to maintain sufficient levels of molecules like ATP, UDP, and pyrophosphate to enable efficient use of the carbon source.

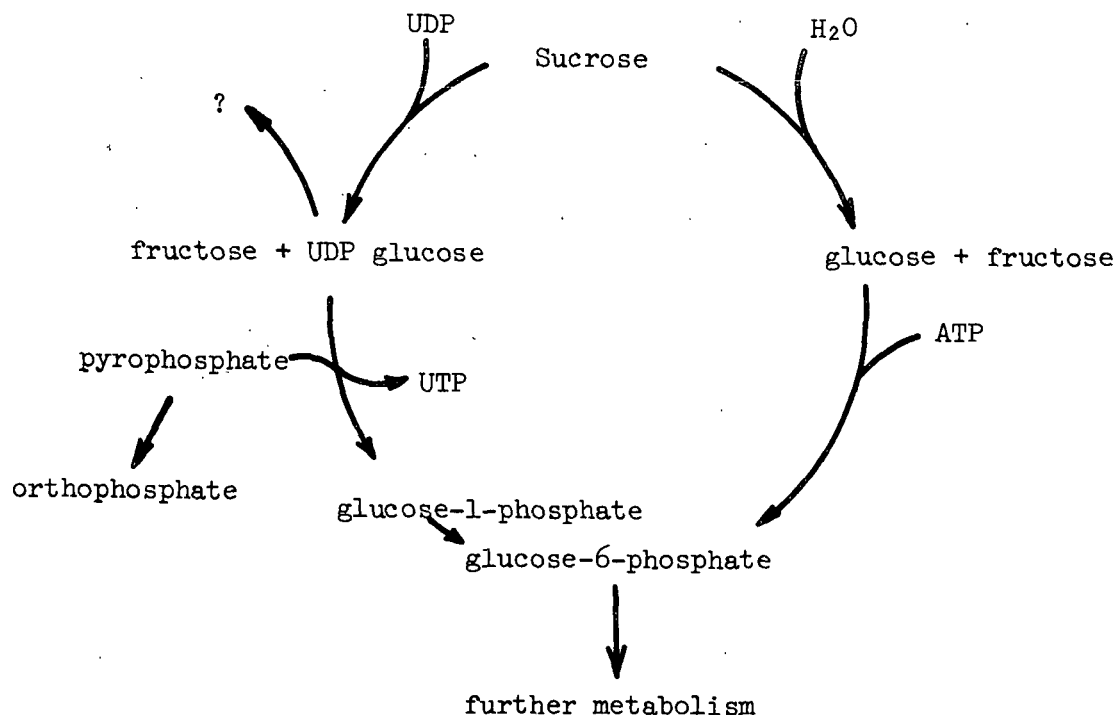


Figure 2. Alternative Pathways for Sucrose Metabolism

# PEROXIDE REGULATION HYPOTHESIS

This hypothesis is based upon the prominence of several enzymes in the cultured cells that are involved in the synthesis, destruction or utilization of hydrogen peroxide. Apparent isozymes of these enzymes have shown some correlation with organizational state. Peroxide analyses to date further suggest a similar correlation. Also, organizational responses have been evoked in feedback experiments by agents that should affect these or related enzyme activities and, on occasion, by hydrogen peroxide itself. Figure 3 is intended to indicate only kinds of reactions that may be determining the effective concentration of hydrogen peroxide at any given time.  $AH_2$  is a general symbol for a reduced organic compound while A is the oxidized form. While peroxide per se cannot be considered to be a specific reagent and its modes of action are not established, one attractive possibility for the action of peroxide is as a crucial factor in the stability of endogenous auxin as detailed under the IAA oxidase hypothesis.

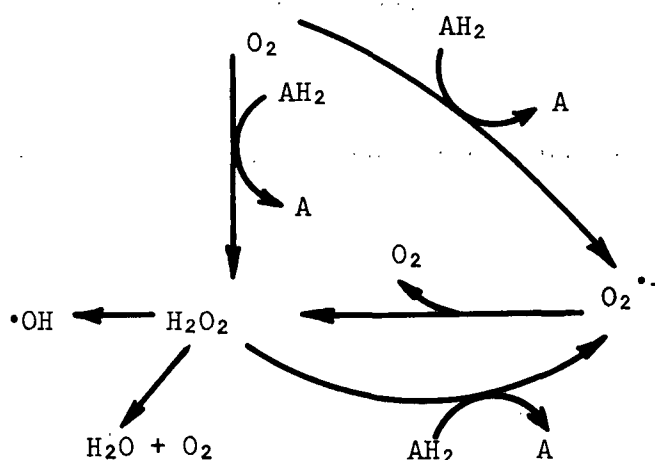


Figure 3. Reactions Determining the Effective Concentration of Hydrogen Peroxide

## IAA OXIDASE HYPOTHESIS

The classical method for induction of organogenesis or embryogenesis in plant tissue cultures is to manipulate the ratio of auxins to cytokinins in the tissues. The capacity of a given system to synthesize and/or degrade these and other plant hormones could easily be a significant factor with which one must contend in attempts at external manipulation of the system. Figure 4 depicts some important reactions relating to natural auxin (indoleacetic acid) in this regard. The relationship with the peroxide regulation hypothesis is quite obvious. All parts of this scheme are under investigation, but the oxidation of IAA by IAA oxidase is receiving the most attention at the moment since it is considered by most investigators to be a form of peroxidase, as our results also indicate. Although our cultures may not be habituated for auxin, they may still contain sufficient quantities of IAA to retard morphogenesis. In its current form this hypothesis is that, due primarily to inactivity of IAA oxidase, endogenous auxin levels are too high to permit morphogenesis. Quantitative analysis of endogenous auxin is in progress.

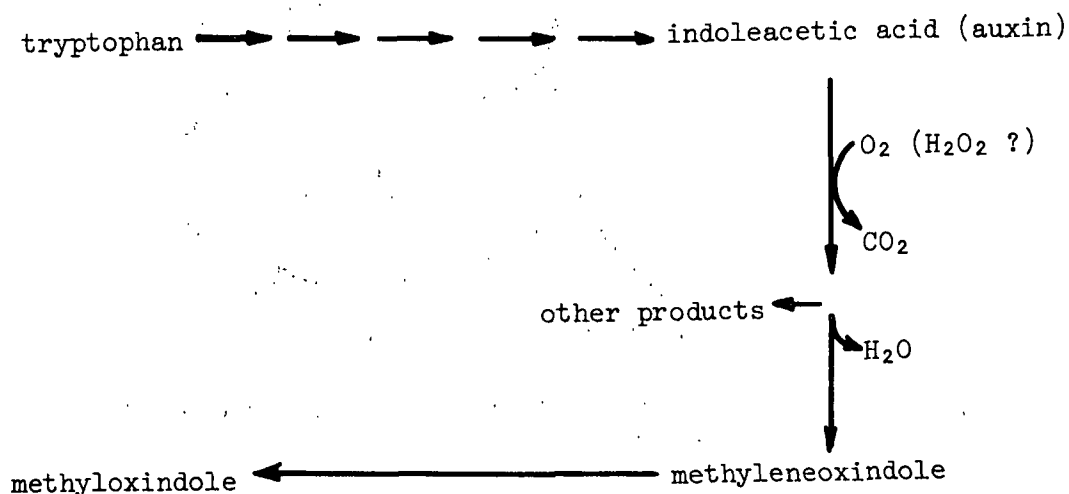


Figure 4. Reactions Important in the IAA Oxidase Hypothesis

## GROWTH AND ORGANIZATION IN CALLUS CULTURES

### NEW NEEDLE CALLUS FROM DOUGLAS-FIR AND LOBLOLLY PINE

#### Introduction

Callus tissue is the basic experimental material for Project 3223 and is used for biochemical analyses, ultrastructural preparations, and for suspension cultures. A constant and adequate supply of callus must be maintained in the tissue culture lab for all phases of the program, and must be fairly uniform and healthy, with dividing cells that are mainly undifferentiated. The short-ranged objective of Phase I is to determine the trigger mechanism of embryogenesis in suspension cultures. This will permit the mass production of plus, elite, superior, and hybrid conifer trees by a commercially-feasible method in Phase II. Since we wish to propagate young trees that have been proven superior, we have selected needle callus as the source for all studies except for a few comparative analyses. Biochemical investigations indicate that needle callus contains enzyme systems that may be involved in embryogenesis, whereas they are absent or weak in stem callus. Seed-embryo tissue contains factors that stimulate organogenesis in callus, but since we do not expect young trees several years old to have seeds, we are developing methods for using needle callus for vegetative propagation from suspensions.

During the past year we changed to needle callus as our experimental material, from a former diversity of callus sources from branches, stems, needles, and seed-embryo cotyledon callus. We have reported the growth of shoots from all of these types of callus except from very old branch callus six years old (Progress Report Four). However, we discarded all old cultures except for a few for biochemical analyses, and protoplasts are produced by students from the original

Douglas-fir callus initiated from branch callus in 1969. Our current policy is to initiate new callus from needles every six months, use it when it is 3-8 months old, then gradually phase it out and discard at 12 months.

Shoots are produced in low frequencies from some cultures during the first to third monthly passage, then the shoot-producing capacity drops off under our environmental conditions for callus growth. This may mean that, as the ability to produce shoots is reduced, so also is the ability to produce embryoids in suspension cultures. As we develop better initiator media, we will attempt to produce embryoids in high frequencies from callus 1-2 months old from initiation. We are also testing now whether shoot-producing callus may be a source of highly-embryogenic cells for transfer directly to developer suspensions. We are awaiting work to be done shortly by Dr. Hsu, in determining hormone levels in callus tissue at different ages from initiation, as well as age from subculture. These data may explain why older callus may lose its embryogenic potential, if natural auxin starts building up in the callus and inhibits morphogenesis.

The purpose of this report is to describe the procedures used in obtaining the needle callus that forms the standard starting point for most of the studies on organogenesis and embryogenesis.

#### Methods and Materials

From young seedlings 1-3 years old in the greenhouse, or from shoots produced from cotyledon, stem or needle callus and grown in aseptic culture, small flushing vegetative shoots were removed when the needles were several centimeters long and surface sterilized in 25-50% bleach for 5 min. After three rinses in sterile water, needles were gently pulled off intact and placed horizontally on the surface of Medium 10, solidified with agar (Appendix Table VII). Petri dishes

were sealed with Parafilm-TM and incubated at 3000-4000 lx of fluorescent Cool White and Sho-Gro illumination, in an incubator on for 16 hr at 24° and dark for 8 hr at 18°C. This gives somewhat better callus than growing in constant lab light at  $24 \pm 2^\circ\text{C}$  as reported previously.

Callus started to grow from the base of needles after 3-5 weeks as soft-to-firm green tissue. After 6-8 weeks each callus piece was 5-8 mm in diameter and was isolated and grown on fresh Medium 10. Each 3-4 weeks, the callus was subcultured and pieces transferred to fresh agar medium. Some callus remained fairly firm during subsequent subculturing, especially in cultures that produced shoots. However, from 20 new cultures, 5-10 usually became softer with age and were kept as stock cultures for suspension studies. Most cultures usually had scattered embryonic cells and an occasional small embryoid, but a few cultures seemed to be without any cells with morphogenetic potential. One culture is now over a year old that continually has had frequencies of embryonic cells ranging from 5-10% and 1-2% small embryoids.

#### Results and Discussion

Given below is the number of callus cultures initiated from needles.

Date	Douglas-fir	Loblolly Pine
1-1-76 to 1-1-77	46	17
1-1-77 to 6-1-77	8	5

We usually maintain 10 cultures of Douglas-fir in bulk of 15-30 dishes and about 10 cultures of fewer dishes. Callus is initiated from 10-12 needles per dish in 3-5 dishes per clone, and after two passages from isolation we usually have 15-20 dishes of the best clones, with five callus pieces per dish. There is no large-volume culture for loblolly pine, but there are several dishes each on several

test media from stem and needle tissue. The stem callus is greener, and is used to improve the medium for needle callus. Several new cultures from needles are light green and seem to be responding to new media being devised by Dr. Waithaka. He has tested many media both related and unrelated to Mrs. Verhagen's LV media reported previously. It is too soon to report on the effectiveness of the new loblolly pine callus media. Both stem and needle callus is being used to study growth in suspension cultures of loblolly pine, although occasional embryoids are also found in the course of the growth studies. Douglas-fir needle callus is being used for empirical and feedback suspension studies for embryoid initiation and development.

### Conclusions

Nutrient media for the continuous culture of loblolly pine needle callus are now being tested, but there are encouraging signs of some increased greening in light-grown callus and also significant growth in suspensions.

We seem to have a satisfactory nutrient medium for initiating and maintaining needle callus from Douglas-fir seedlings 1-3 years old. Medium 10 also was satisfactory for callus culture of stem, branch, seed-embryo and seedling parts of Douglas-fir when they were grown in the lab. We were not able to improve callus growth by reducing iron in the medium, but for some cultures the growth was faster and callus softer when regular Bacto-agar was used. In many cases, continuous growth on Noble agar resulted in slower growth and frequently softer and darker-green callus. This often accompanied more embryonic cells. However, browning often occurred at the edges. In the few cultures benefiting from Noble agar, we are continuing its use to produce cultures of softer callus with cells that dissociate easily. This callus makes the best source for suspensions where screening for small cell clusters is being tested. We also feel that our present



environmental balance of light and temperature is satisfactory for good callus growth. The environment and Bacto-agar tends to reduce the natural embryogenesis in callus that we have observed when callus is grown in the lab. At this time we want undifferentiated cells to start suspensions, in order to demonstrate that embryogenesis does, in fact, occur in liquid suspensions. We have recently been able to do this by starting with callus that does not contain any morphogenetic cells, and then watching the formation of new embryonic cells and embryoids in suspension. This will be discussed later.

The initiation of many clones, and the selection of only the best, should insure that we always have an adequate supply of soft green callus, composed either entirely or mostly of undifferentiated cells. Concentration on only one type of callus (needle callus) should also reduce the total number of cultures carried at one time, giving more space for fewer and better clones. This should avoid confounding results caused by using different types of callus from stem, etc.

#### Future Plans

We plan to start new needle callus every 1-2 months and start selection for soft and green callus at the end of the second passage after isolation. Any shoot production should have occurred by this time. Additional methods of increasing the supply of soft green callus are increasing the level of auxin in the medium, subculturing callus regularly after three weeks instead of the usual four weeks, and reducing the light level in the incubator. These factors will be tested in coming months. These tests are implied in the timetable (Appendix Table VI) under Callus Cultures in Section I. We will continue to collect needles from seedlings of different ages in the greenhouse, but do not plan to collect from older trees on the Institute grounds until natural flushing occurs next spring.

## SHOOTS FROM NEEDLE CALLUS OF DOUGLAS-FIR

### Introduction

The major objective of Phase I of this program has always been embryogenesis in suspension cultures. However, we have produced shoots from whole seed embryos, cotyledons and cotyledon callus, as well as from stem and needle callus of young Douglas-fir seedlings. This was reported in the last annual report and has just been published (3). We continue to find shoot production from newly-initiated needle callus on regular nutrient Medium 10, but we make no special effort to deliberately produce shoots from callus.

Our first shoot from subcultured cotyledon callus was found November 18, 1974, and subsequent shoot production from cotyledons and callus was reported in Progress Reports Three and Four, 1976. Our first shoot from subcultured needle callus of Douglas-fir was found January 2, 1976, and was reported in Progress Report Four.

Shoots from subcultured cotyledon callus appeared during the third to sixth passages, but shoots from subcultured needle callus have consistently appeared during the first few passages after isolation. We designate isolation as the first subculture. Shoot production from needle callus has been unspectacular. However, as many shoots as possible have been isolated and allowed to elongate for biochemical analyses and needle cultures. Recently, we started to test shoot-producing callus as a possible source of highly-embryonic cells for suspension cultures, where embryonic cells may develop into embryos and plants. If this becomes a reality, then we will indeed become excited about the production of shoots from needle callus.

The purpose of the following section is to report the occurrence and frequency of shoot production from needle callus.

#### Method and Materials

Seeds from Seed Lot 491-15-1 were surface sterilized in 50% bleach for 15 minutes and rinsed three times in sterile water. Embryos were then removed from the Douglas-fir seeds and placed on Medium E-1 (Appendix Table VII) containing 0.1 mg/liter BAP (benzylaminopurine). In the light incubator the cotyledons produced multiple shoots which were excised and grown on Medium E-1 without hormones. The shoots developed normally after three months, and needles were removed and placed on Medium 10 in the light incubator having 16 hours of 5,000 lx fluorescent light at 24°C, alternating with 8 hours darkness at 18°C. Callus grew from the base of the needles collected from young shoots grown aseptically, as well as from seedlings grown in the greenhouse, and was isolated and subcultured monthly to fresh Medium 10.

#### Results and Discussion

Table I lists 13 occasions of shoot initiation from Douglas-fir needle callus in our lab. Only 18-20 individual shoots have been recovered over a period of 17 months in 1975-77. The two cases of multiple shoots each had dozens of small shoots coming from the same meristematic region. Most shoots were isolated and grown on medium either lacking or having a low concentration of auxin. All shoots elongated except those from clones 24 and 27. These were initiated on a variation of Cheng's (4) CI callus-initiation medium, and never elongated past the bud stage when transferred. Shoots were initiated during the first-third subcultures, with one shoot found during the fourth passage, and the two cases cited above that didn't elongate were initiated on callus still attached to the needles. None of the

needle-callus shoots have rooted; however, no special treatments have been tried that might result in rooting.

TABLE I

SHOOTS INITIATED FROM DOUGLAS-FIR NEEDLE CALLUS

Clone No.	Callus Initiation	Subculture Date	Shoot Initiation	No. Subcultures	Pieces With Shoots/Total
72	8- 2-75	12-22-75	1- 2-76	3	--
104	8-10-76	10-15-76	11- 5-76	1	--
13	8-20-76	11- 8-76	12- 6-76	1	3/12
			12-10-76	1	4/12
20	12- 7-76	3-10-77	3-16-77	2	Multiple <sup>a</sup>
		4-14-77	4-25-77	3	2/100
		5- 2-77	6- 1-77	4	--
28	12-23-76	2-28-77	3-16-77	2	1/12
24	12-21-76	--	3-15-77	0	2
27	12-21-76	--	3-15-77	0	1
21	12- 7-76	3-14-77	3-15-77	2	Multiple
		4-11-77	5-11-77	3	--
34	1- 4-77	4-19-77	5-11-77	2	2/8

<sup>a</sup>Multiple shoots indicates one growth center on one callus piece with several dozen shoots.

One might be tempted to say that shoots generally arise in winter and spring from needle callus initiated in winter. However, to date we have not initiated callus every month of the year. During our work with aspen, we seemed to get shoots in the winter and spring from callus initiated in the fall, but were never able to fully document this pattern to the exclusion of others. The

limiting factor for the initiation of needle callus is the availability of flushing needles from seedlings in the greenhouse or from young shoots in the lab. There does seem to be a cycle of flushing, so the rest of the sequential events may merely reflect the cyclic time of flushing. This could mean that we may be limited in the times we can initiate needle callus, unless we can initiate callus from older needles equally well. From our studies, we can get callus from older needles, but not as much, and we have no data on relative quality and quantity compared to flushing needles. Our impression is that we get better callus for suspensions from flushing needles, but can use older needles if we must.

### Conclusions

In past reports we have detailed several studies in which we attempted to duplicate Cheng's method of producing high frequencies of shoots from callus. Cheng was originally using cotyledon callus, and most of our early work was also with cotyledon callus. However, with both cotyledon callus, and more recently with needle callus, we were not able to confirm Cheng's method in our lab. We recover a few shoots from needle callus of Douglas-fir during routine culturing, and so far this is sufficient for our present needs. Therefore, we do not intend to extend our studies of shoot production (organogenesis) from callus, but plan to emphasize embryogenesis from callus or suspension cultures.

### Plans

We will not attempt deliberate shoot initiation from needle callus. However, we will use the casual shoots initiated during routine culturing for biochemical analyses and as a source of aseptic needles for further callus initiation. We will also use shoot-producing needle callus as a possible source for highly-embryonic cells for initiating high frequencies of embryoids in suspension cultures. If this can be done, we will then determine whether we should deliberately try to

increase the frequency of shoot production, or if the routine low frequency is sufficient for our goals. Whatever the result, at present we do not plan to study the mechanism of organogenesis in callus, but rather plan to continue with the original objective of studying the mechanism of embryogenesis in callus and suspension cultures, and the development of embryoids into embryos in suspensions. These plans are reflected in the lack of an entry in Appendix Table VI indicating needle callus shoot initiation studies.

#### GROWTH MEDIUM FOR LOBLOLLY PINE CALLUS

##### Introduction

One of the long-range goals of this project is to be able to propagate superior conifer species and hybrids by embryogenesis in suspensions. Since the pines are extremely important both as a source of pulp and timber, loblolly pine has always been one of our major species of interest. The problem has been that we cannot culture loblolly pine callus as well as Douglas-fir callus. Loblolly pine callus grows well in the dark, but soon loses its ability to produce chloroplasts. On the other hand, Douglas-fir callus will not survive in the dark. For the past two years we have been spending minimum time in the lab in improving the nutrient and environmental factors associated with loblolly pine callus culture. Most of our time has been spent working with Douglas-fir. Shirley Verhagen has developed a minimum medium for callus growth, which is now the control for a study just started on growth in suspensions of loblolly pine, by Kimani Waithaka, our new tissue culture Post-Doctoral Fellow. This section reports the early work of Verhagen in developing the minimum nutrient medium. Waithaka's new study on growth in suspensions will be reported under new studies.

At the start of this project, we concentrated on initiating callus from cambial or cortical tissue in terminal branches of trees 5-15 years old. Stem callus was thus initiated from parental explants between 0.5 and 1 cm in diameter. This method was satisfactory for Douglas-fir, but loblolly pine callus did not grow well when isolated from the explants and grown independently on either Medium 3 or 10 (Appendix Table VII). Usually the callus was initiated as light-green tissue on Medium 10, but turned yellow and then brownish yellow after a few subcultures.

Currently, we are initiating callus from needles of both Douglas-fir and loblolly pine, after needles are removed from young seedlings 1-2 years old. However, we were able to significantly improve the growth of stem callus initiated from terminal branches of 3-year-old loblolly pine trees a year ago and still maintained in culture. Positive results are required, because high-quality stem callus is now an important source of cells for the new suspension program. The new method of initiating callus from needles was reported in the first section of this report. Loblolly pine callus used here is from terminal branches, and was initiated by the method of Winton (5), and is also referred to as stem callus.

The purpose of the comments that follow are to update interested individuals with the problems associated with initiating and growing loblolly pine callus and to discuss recent progress in solving the associated problems.

#### Methods and Materials

Terminal branches were received from International Paper Company, collected from 3-year-old loblolly pine trees growing in a test plot. Callus was initiated and subcultured to Medium 10, where it changed from a light-green friable callus to brownish-yellow tissue over several passages. As part of our program to develop

an optimum nutrient medium for loblolly pine stem callus, new callus was distributed among several new media adapted from Medium 10. Not only were hormone and chemical additives tested, but also the light quality and quantity were varied.

### Results and Discussion

A general result of the growth tests is that loblolly pine callus apparently grew better with higher levels of auxin than does Douglas-fir. In some cases, 2,4-D seemed to do as well as does the regular NOAA (naphthoxyacetic acid). Of course, as with Douglas-fir, cotyledon callus grew faster than stem or needle callus, confirming IPC biochemical analyses that the seed cotyledon callus contains different chemicals, possibly in the form of nutrients intended for the developing seedling. Cotyledon callus grew better and faster than needle callus over many months, indicating that the advantages of cotyledon tissue persist in subcultured callus. However, the main criteria for our optimized loblolly pine medium is the increased greening of needle callus and the reduction in cell size to approximately that of Douglas-fir callus cells.

Needle callus from the 3-year-old trees is now generally growing better on LV-10 medium than on Medium 10, but the results are not always the same for each passage. Sometimes a higher phosphorous level seems better with lower calcium; then, during other passages the difference is not as noticeable. We still cannot decide quantitatively whether the addition of tryptophan increases or inhibits growth. LV-10 medium is shown in the Appendix Table VII.

In the new study of growth in loblolly suspensions, stem callus is available in fair quantities for preliminary experiments. However, the most promising new growth media will be tested again with needle callus for fine adjustments and to determine clonal variation.



### Conclusions

Verhagen worked on the LV medium for about two years, and even at the relatively low intensity of effort she has developed a medium that is minimally satisfactory for callus initiation and maintenance. Needle callus starts well on both Medium 10 and on LV-10, but probably survives better on LV-10. Preliminary tests in growth suspensions indicate that LV-10 has not yet been improved as a growth medium for certain clones in suspension cultures.

### Future Plans

Future improvement of agar medium for loblolly needle callus will be guided by current studies of growth of loblolly pine suspension cultures. Verhagen will not pursue further independent experiments at this time, but may test feedback suggestions from Waithaka, based on his suspension results. The study to improve loblolly pine callus medium by Verhagen has thus been completed.

## ENZYME INVESTIGATIONS

### Introduction

All enzyme research reported in this section is related to either the peroxide regulation hypothesis or the IAA\* oxidase hypothesis. These two hypotheses (described in detail under Working Hypotheses) are strongly related to each other, and, as our investigations continue, it may become more reasonable to consider the IAA oxidase hypothesis subsidiary to the peroxide regulation idea or vice versa. Although our current data do not permit subscription to the notion (6) that all isoperoxidases also have IAA oxidase activity, we do see correspondence for some anionic species. Each of the enzymes under discussion here relates in some way to hydrogen peroxide, its production, destruction, or utilization; therefore, the

---

\*See list of abbreviations in appendix.

rationale for the study of these enzymes relative to a developmental hypothesis based upon regulation of peroxide concentration is fairly obvious. The balance among these and a few other enzyme activities should determine the effective concentration of peroxide in vivo at any given time. In turn, combinations of specific enzymes and oxidants (e.g., peroxide) may determine effective levels of constituents involved in development, endogenous auxin (IAA) being the prime example currently under scrutiny. Consequently, we are studying distributions and interrelationships of these enzymes and their various forms (isozymes) as a function of organizational state. Also, it is of importance to learn whether these relationships are affected by environmental manipulations such as changes in standard medium components and special additives, e.g., hormones or various feedback treatments, or by endogenous changes in organized and unorganized tissues.

Specifically, the enzyme investigations reported here sought to establish the isozyme distribution patterns for IAA oxidase in organized and unorganized conifer tissues and then to compare these distributions with those of the isoperoxidases. This comparison with peroxidase was essential because of the postulated close relationship between the two enzymes and the fact that we were already in possession of considerable data indicating that isoperoxidase patterns may correlate with morphogenetic potential or organizational state. Furthermore, the literature on isoperoxidases in other plants also indicates that they can be correlated with embryogenesis (7). Our comparison goes beyond electrophoretic behavior to such properties as substrate and cofactor requirements of which the peroxide involvement is the major example in this report.

In addition to the major emphasis on IAA oxidase and peroxidase relationships, these and other enzymes involved in hydrogen peroxide metabolism were examined for their responses to plant hormones. The IAA oxidase and peroxidase

isozymes of loblolly pine were studied in all but needle callus for comparison with Douglas-fir results. Also probed to a limited extent was the question of the extracellular localization of some of these enzyme activities.

The purpose of the discussion that follows is to update interested individuals in the status of enzyme investigations and report the degree of correlation being found between enzyme activity and morphogenesis. The overall relationship between the enzyme investigations, including the time frame involved, and the other studies that are considered to be important to the peroxide regulation hypothesis is given in Appendix Table VI.

#### Methods

With the exception of on-gel staining for IAA oxidase following disk electrophoresis, procedures used were noted in previous reports (see Progress Report Four, p. 29). For IAA oxidase detection on gel, the procedure of Endo, as described by Srivastava and van Huystee (8), is now employed at pH 3.6. Higher pH's have been used but, although activity is evident, excessive background develops as one proceeds toward the alkaline side.

#### Results

##### Peroxidases

Considerable information on these activities was presented in previous reports. However, Fig. 5 and 6 show how Douglas-fir stem callus isozymes respond to in vivo treatments with 2,4-D and BAP as recorded by anionic and cationic electrophoresis, respectively. Treatments have also been conducted on gel following electrophoresis but prior to staining; those results (not shown) indicate little effect. Considering the data in Fig. 5 and 6, along with other results not presented, 2,4-D does seem to enhance the fast moving peak to the far right

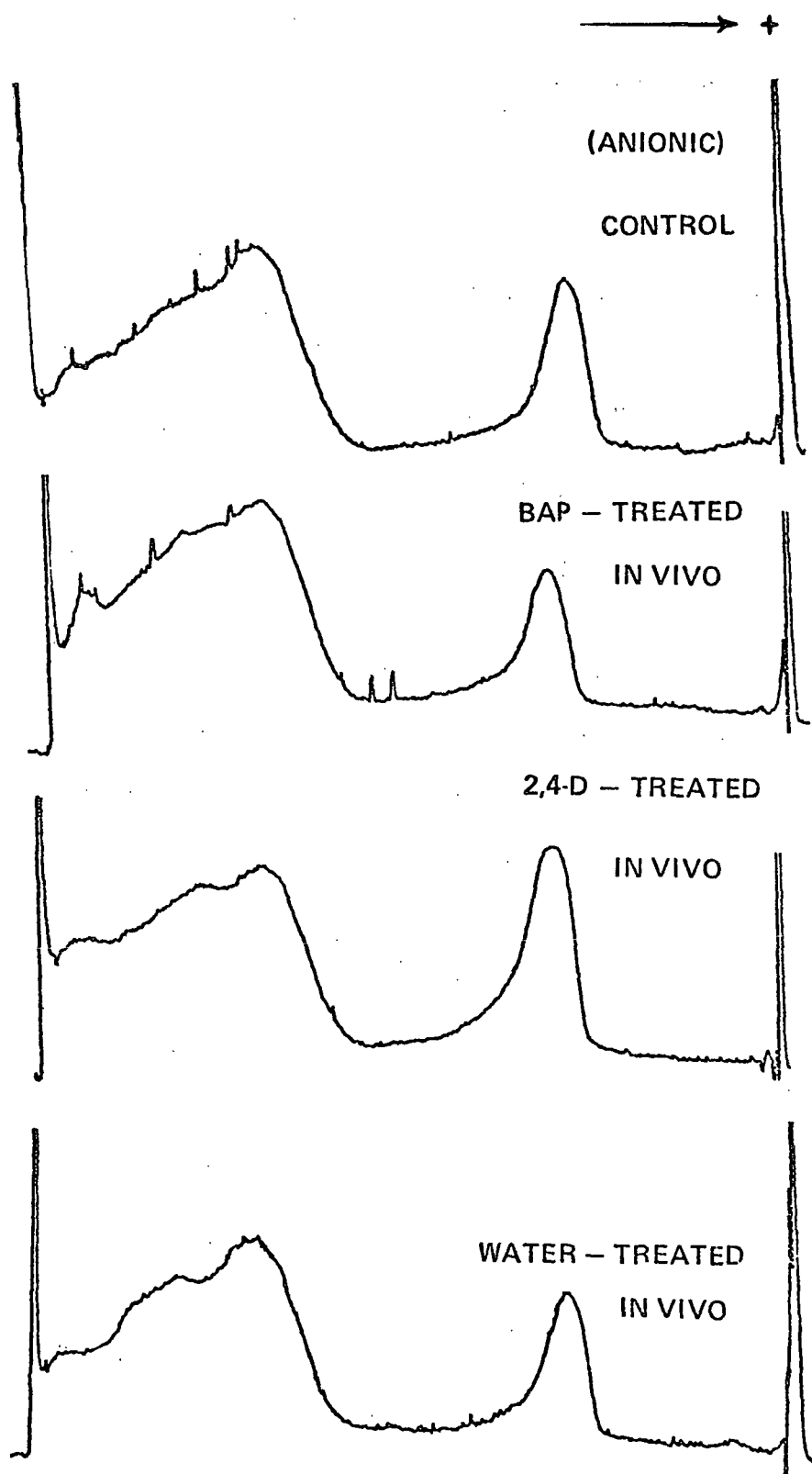


Figure 5. Anionic Electrophoresis of Extracts from BAP and 2,4-D Treated Douglas-fir Stem Callus. Peroxidase Activity. BAP and 2,4-D at 5 mg/liter, 3 Days

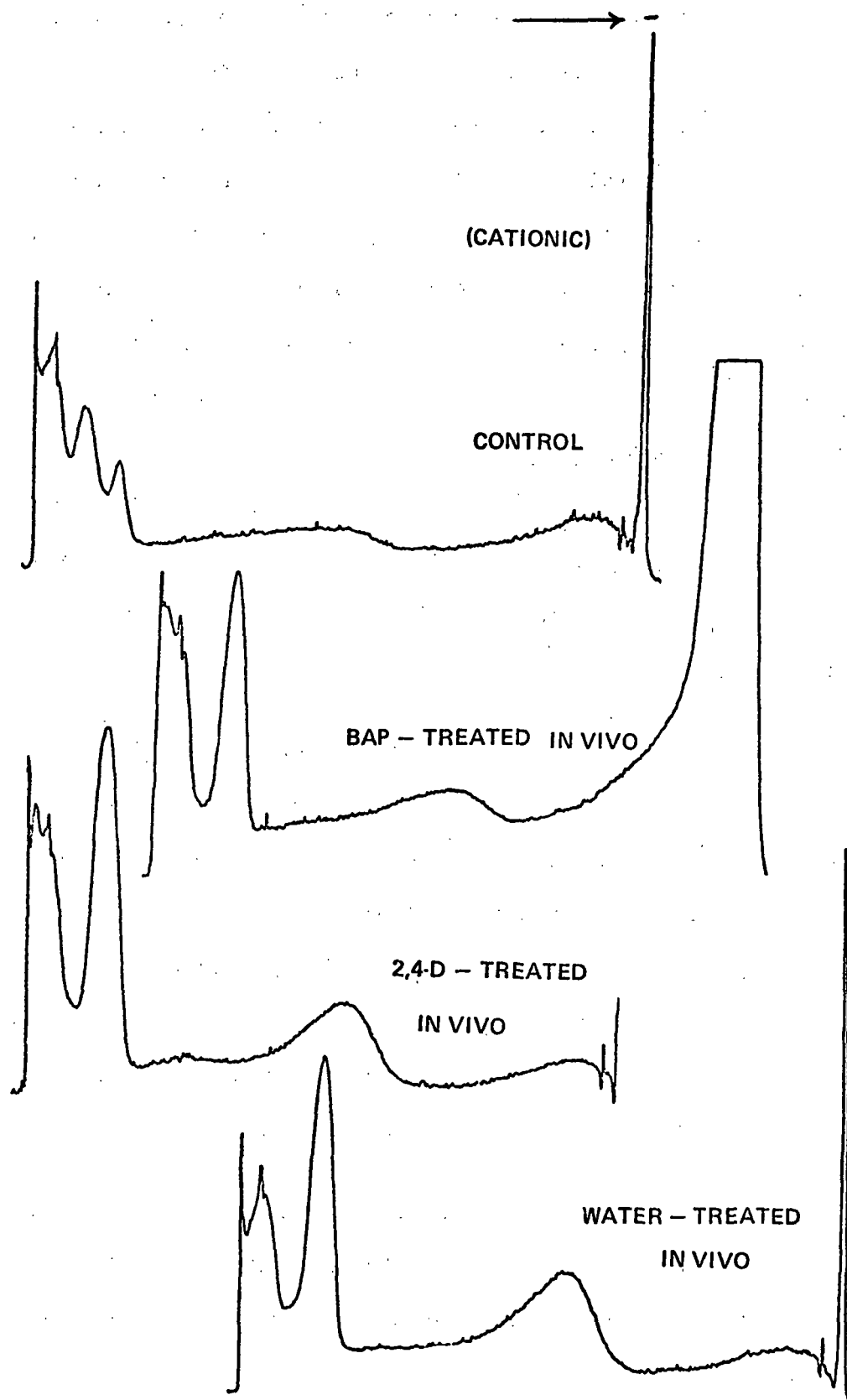


Figure 6. Cationic Electrophoresis of Extracts from BAP and 2,4-D Tested Douglas-fir Stem Callus. Peroxidase Activity. BAP and 2,4-D at 5 mg/liter, 3 Days

in anionic electrophoresis. Lee (9) observed a similar response in the case of cultured tobacco. BAP may affect a very slow moving anionic species and a very fast moving cationic species; the on-gel treatment (not shown) tends to agree with this. We have some indication that the very fast moving cationic species (far right) may be an artifact. The responses show some similarities to the results of others who have treated plants with auxins and cytokinins followed by examination of effects on isoperoxidases (9,10). The overall results suggest that the 2,4-D and BAP had little direct influence on peroxidase activities in Douglas-fir callus extracts in vitro. However, the in vivo responses show that their hormonal effects could be mediated through production or suppression of peroxidase isozymes. This means that 2,4-D and BAP should have long-term indirect hormonal influence on isoperoxidases, but no immediate effects upon isoperoxidases would be expected upon addition of 2,4-D or BAP.

The activity of isoperoxidases was also examined with diaminobenzidine (DAB) as substrate in extracts of loblolly pine seedlings and callus tissues. The anionic traces for isoperoxidases in extracts of loblolly cotyledon and stem callus grown on Medium 10 are presented in Fig. 7; the cationic traces are in Fig. 8. The anionic and cationic traces for loblolly seedling sections appear in Fig. 9 and 10, respectively. One can see considerable similarity with Douglas-fir callus patterns. As of this writing, data on the isoperoxidase patterns from loblolly pine needle callus extracts are not yet available.

It has also been found that peroxidatic enzyme activity is released into suspension medium during the culture of Douglas-fir stem callus tissue (Fig. 11). What has previously been referred to as DAB oxidase (Progress Report Four, p. 50) also shows up.

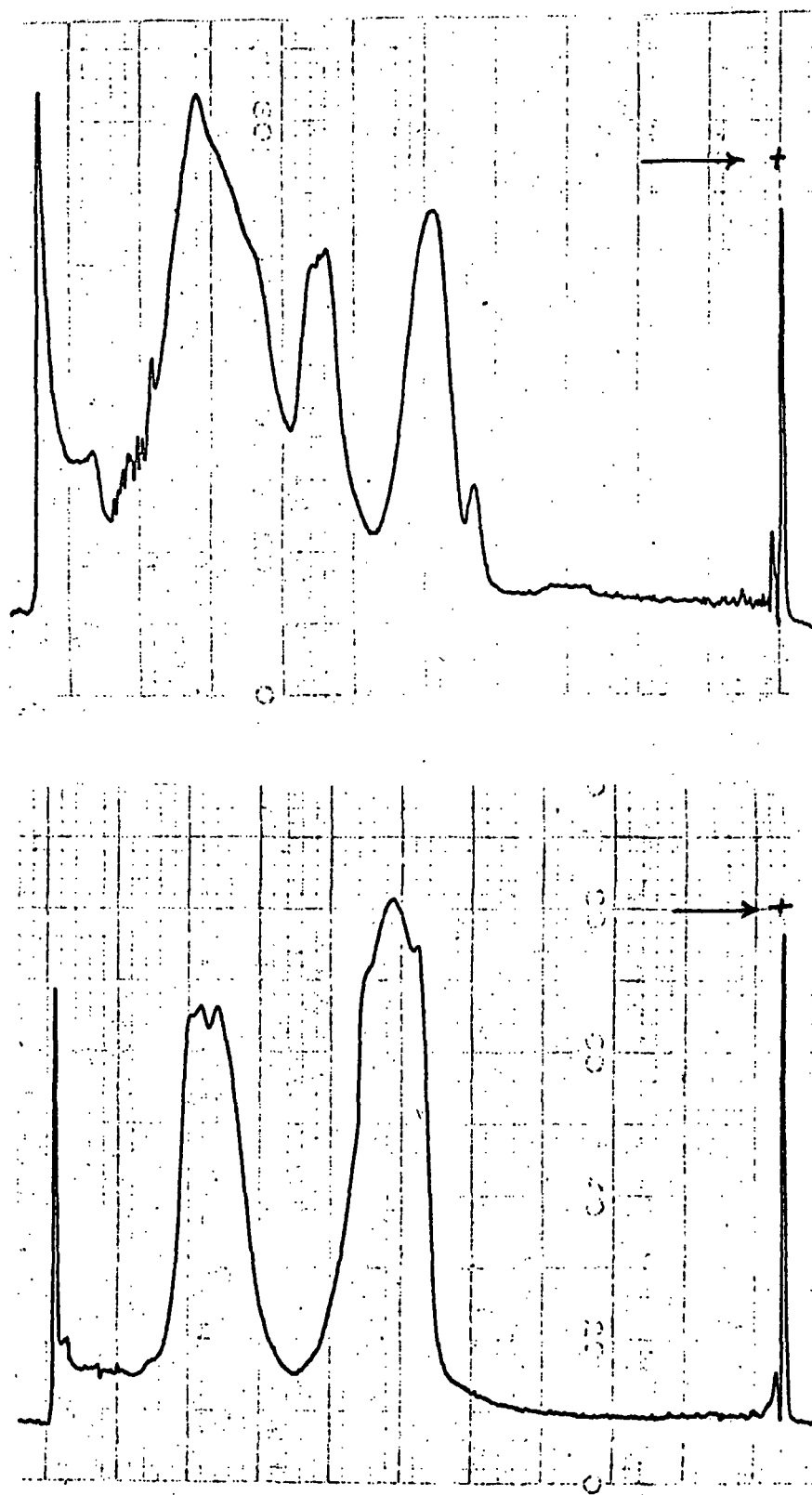


Figure 7. Anionic Electrophoresis of Extracts from Loblolly Pine Callus. Peroxidase Activity. Top: Cotyledon Callus. Bottom: Stem Callus

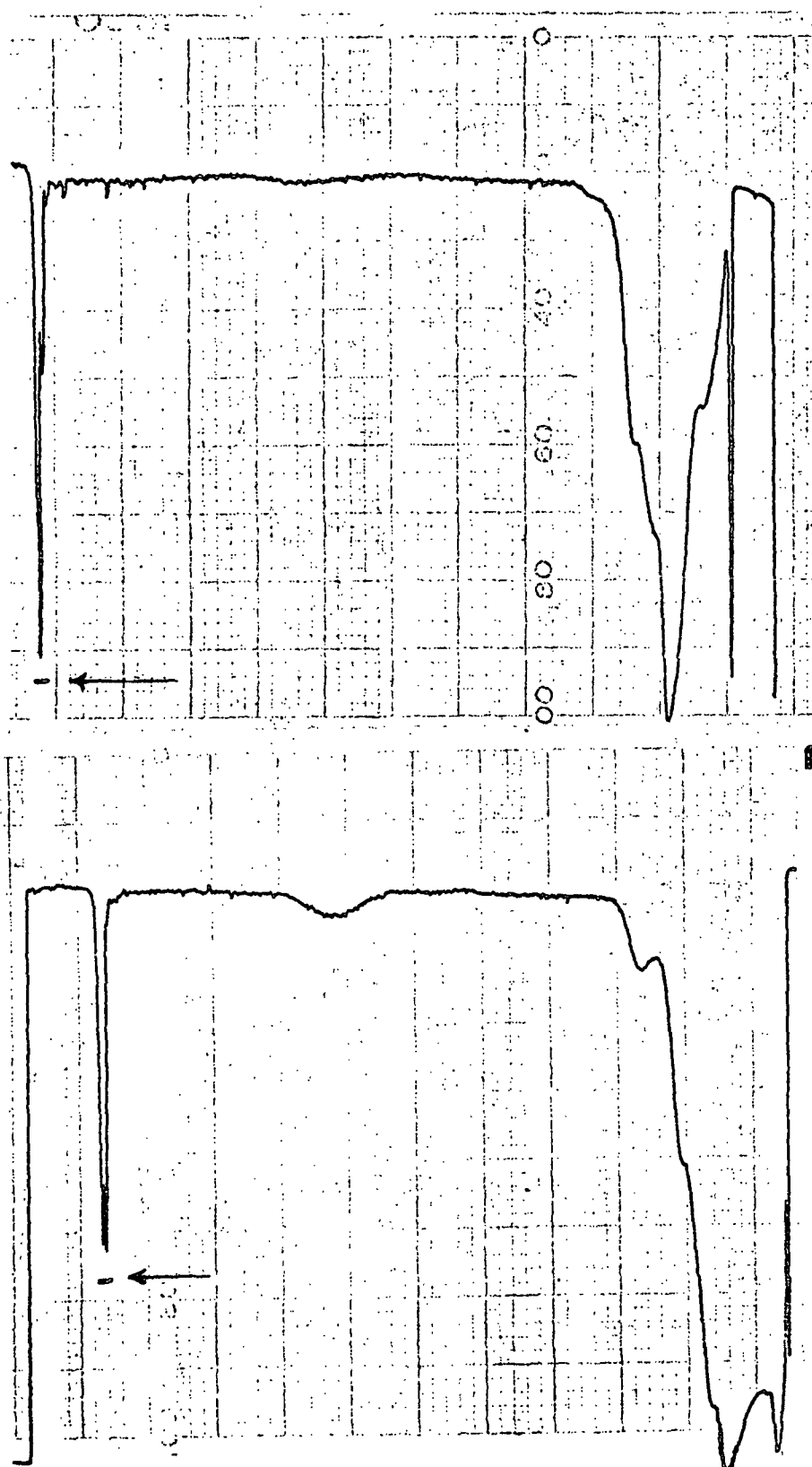


Figure 8. Cationic Electrophoresis of Extracts from Loblolly Pine Callus.  
Peroxidase Activity. Top: Cotyledon Callus. Bottom: Stem  
Callus



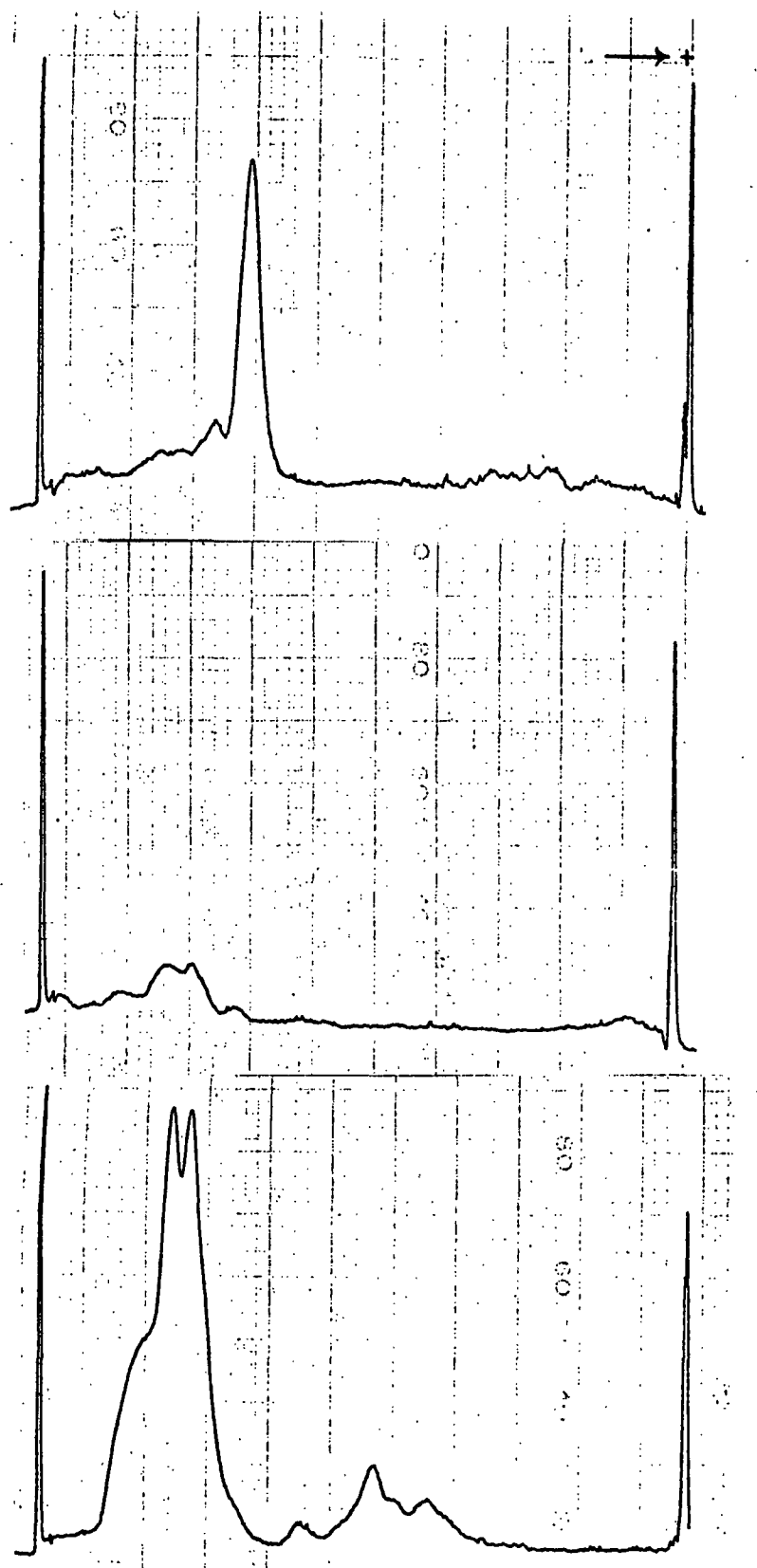


Figure 9. Anionic Electrophoresis of Extracts from Loblolly Pine Seedling Tissues. Peroxidase Activity. Top to Bottom: Needles, Stems, Hypocotyls-Roots

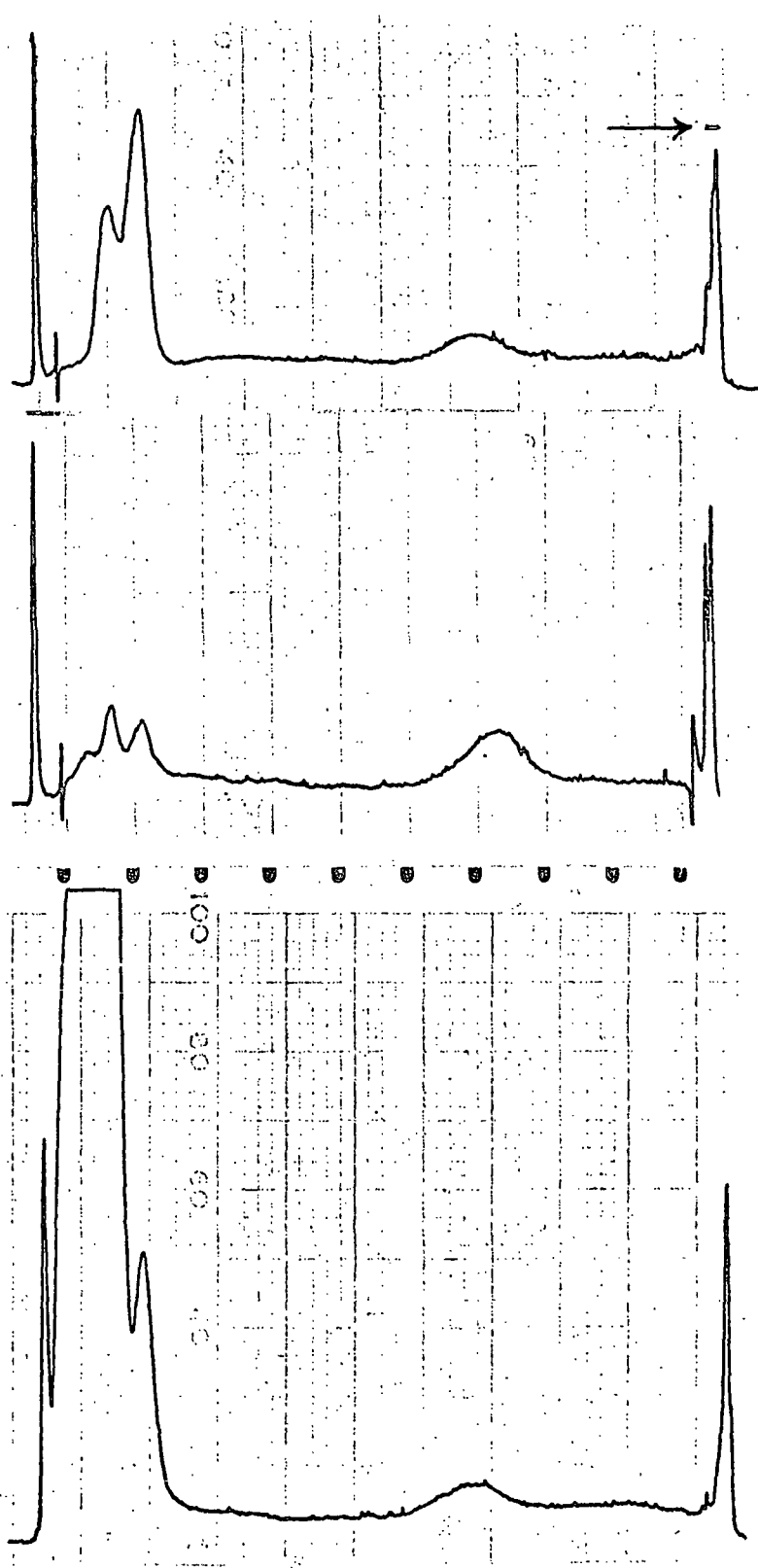


Figure 10. Cationic Electrophoresis of Extracts from Loblolly Pine Seedling Tissues. Peroxidase Activity. Top to Bottom: Needles, Stems, Hypocotyls-Roots

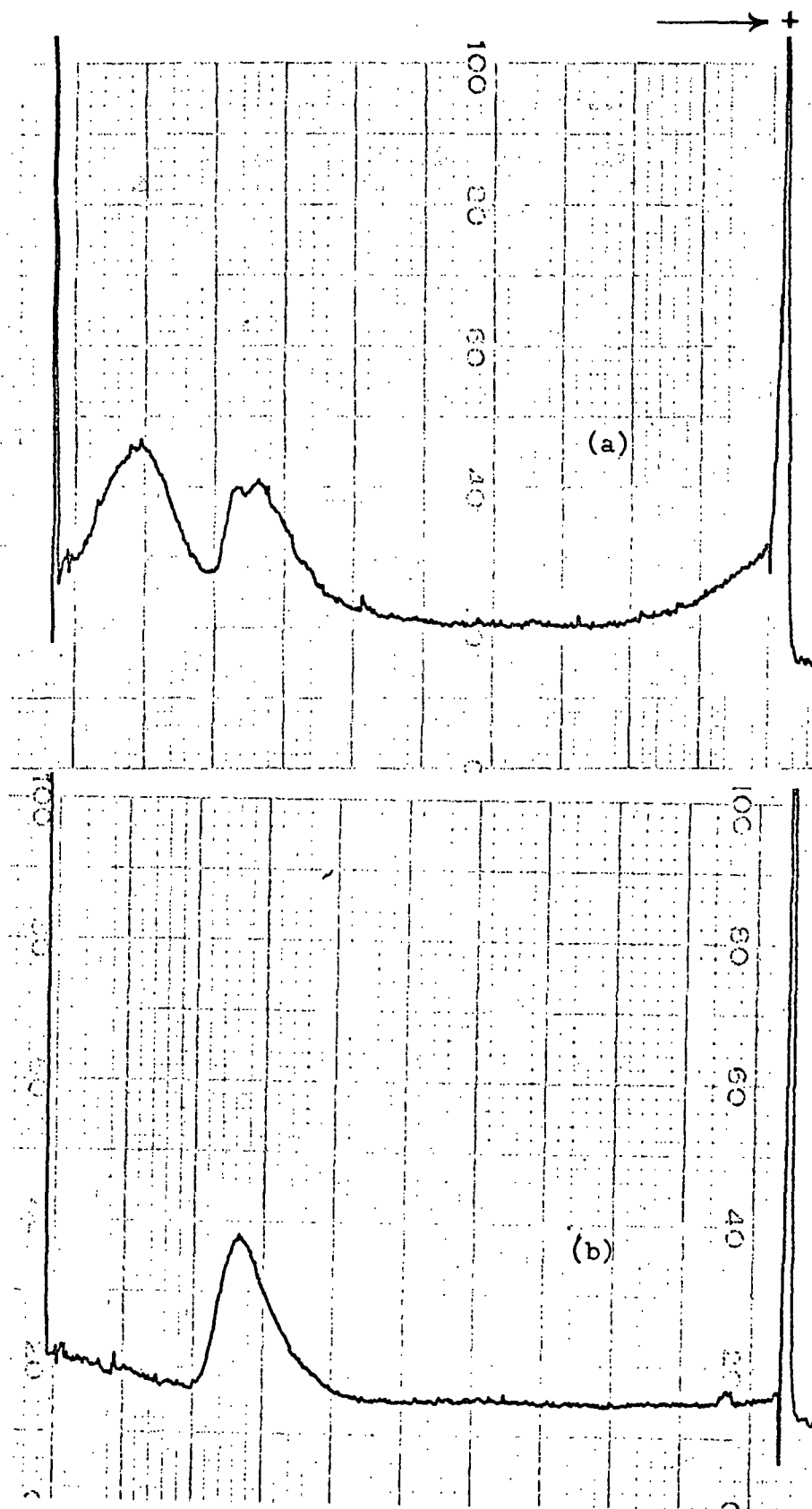


Figure 11. Anionic Electrophoresis of Cell-free Suspension Medium After Culture of Douglas-fir Stem Callus Cells. Peroxidase Activity (a) and DAB Oxidase Activity (b)

### Superoxide Dismutase

The effects upon this enzyme of treating Douglas-fir stem callus with 2,4-D and BAP in vivo and with BAP on-gel following anionic electrophoresis are shown in Fig. 12 and 13, respectively. Recalling that peaks are inverted in this assay, note that the major effect is the increase in the major peak of Fig. 13 by BAP which is the opposite of the water treatment. It will also be noted that the untreated controls are quite different in Fig. 12 and 13; the only experimental difference was that the callus used to obtain the data in Fig. 12 was two weeks older than in the case of Fig. 13 (this has been noted previously, e.g., Progress Report Four, p. 45). Cationic electrophoresis runs for these treatments also show some effects (not shown). Results available at this time indicate that the plant hormones interact directly with this enzyme. Therefore, in contrast to the isoperoxidase case, rapid responses to BAP could involve superoxide dismutase.

### IAA Oxidase

While this activity has been of interest for a long time, the on-gel assay used previously was not satisfactory (e.g., Progress Report Four, Fig. 22). The use of Endo's procedure soon revealed that some bands of peroxidase in Douglas-fir stem callus extracts also have IAA oxidase activity.

Anionic disc electrophoretic distribution patterns of isozymes of peroxidase and IAA oxidase in extracts of Douglas-fir stem callus are compared in Fig. 14. Isoperoxidases are typically found in three regions of anionic electrophoretic mobility although each of these regions exhibits peaks that are not homogeneous, i.e., more than one isoperoxidase is probable per peak. In contrast, in stem callus extracts most of the IAA oxidase activity is associated with only one of these regions, but again there are IAA oxidase isozymes within that region. Correspondence of isoperoxidases with isozymes of IAA oxidase is apparent in these

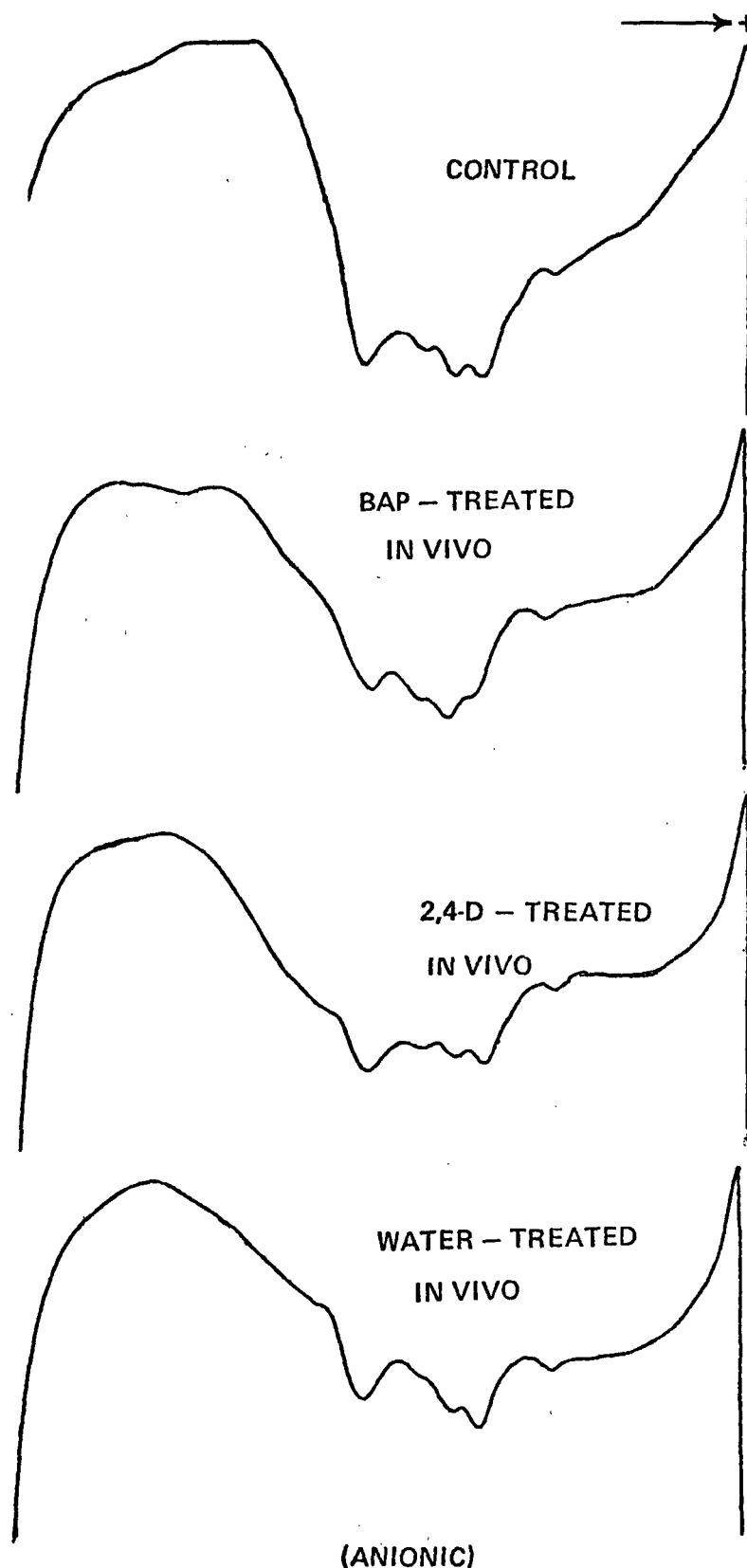


Figure 12. Anionic Electrophoresis of Extracts from BAP and 2,4-D Treated Douglas-fir Stem Callus. Superoxide Dismutase Activity. BAP and 2,4-D at 5 mg/liter, 3 Days

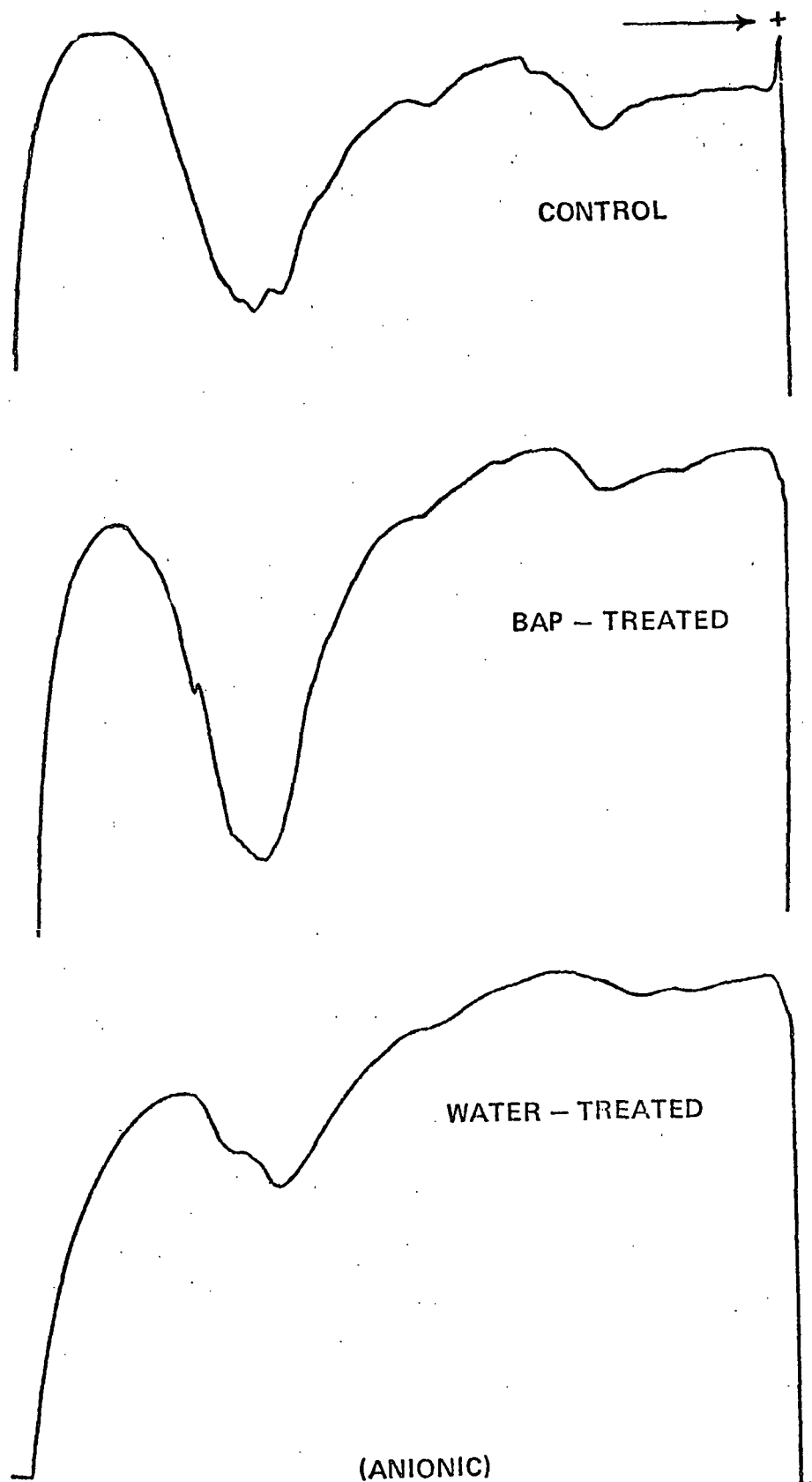


Figure 13. Anionic Electrophoresis of Extracts of Douglas-fir Stem Callus Treated After Electrophoresis with BAP. Superoxide Dismutase Activity. BAP at 0.5 mg/liter, 20 Min

patterns. A similar isoperoxidase pattern for an extract of shoot-producing Douglas-fir needle (not cotyledon) callus is given in Fig. 15. Note that IAA oxidase activity appears to be associated with all of the isoperoxidases in this case.

Figure 14. Anionic Electrophoretic Distribution Patterns of Peroxidase (top) and IAA Oxidase (bottom) Extracted from Douglas-fir Stem Callus

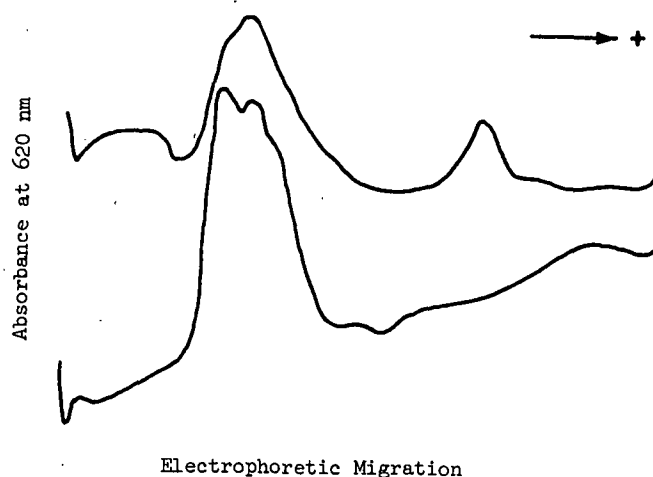
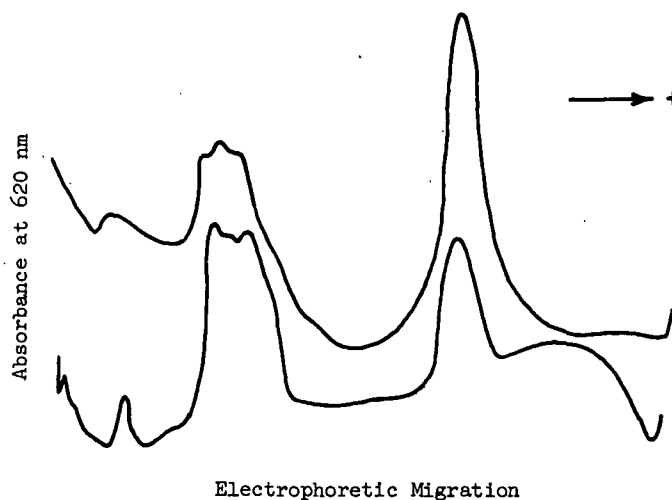


Figure 15. Anionic Electrophoretic Distribution Patterns of Peroxidase (top) and IAA Oxidase (bottom) Extracted from Shoot-producing Douglas-fir Needle Callus



Activities of IAA oxidase and peroxidase isozymes were also examined in extracts of organized tissues, i.e., from different parts of greenhouse seedlings. A distinct anionic isoperoxidase pattern is found to be associated with each of the seedling sections: needles, stems, and hypocotyls-roots. Anionic IAA oxidase distribution patterns again show considerable correspondence with the peroxidase

patterns. The most useful information gleaned from studies on the isozymes of peroxidase and IAA oxidase thus far relates to the hydrogen peroxide requirements of these enzyme activities. Anionic isoperoxidase patterns of Douglas-fir needles, stems and hypocotyls-roots are shown in Fig. 16-18 in the presence and absence of added hydrogen peroxide. In each case it can be seen that some isozymes appear even in the absence of added peroxide. Although IAA oxidase activity is strongly associated with isoperoxidases, the oxidizing agent is not generally considered to be hydrogen peroxide in the case of IAA oxidation; however, it is often added as a promoter of IAA oxidase (11). It may be notable, therefore, that IAA oxidase activities from the various parts of the seedling (Fig. 19-21) show complete dependence upon peroxide addition. The dependence of some isoperoxidase activity and all IAA oxidase activity upon added hydrogen peroxide is also found in callus extracts as shown for Douglas-fir needle callus in Fig. 22.

Figure 16. Anionic Electrophoretic Distribution Patterns of Peroxidase Extracted from Douglas-fir Needles and Assayed in the Presence (top) and Absence (bottom) of Added Hydrogen Peroxide

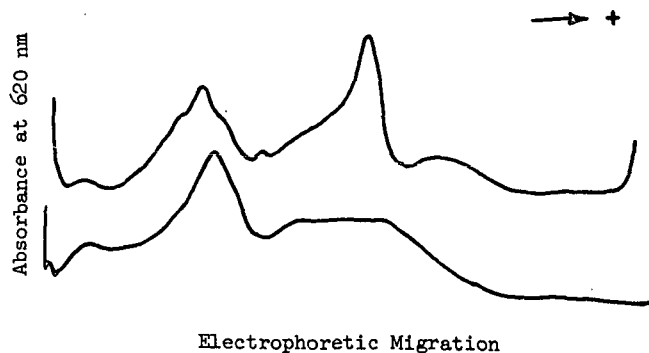


Figure 17. Anionic Electrophoretic Distribution Patterns of Peroxidase Extracted from Douglas-fir Stems and Assayed in the Presence (top) and Absence (bottom) of Added Hydrogen Peroxide

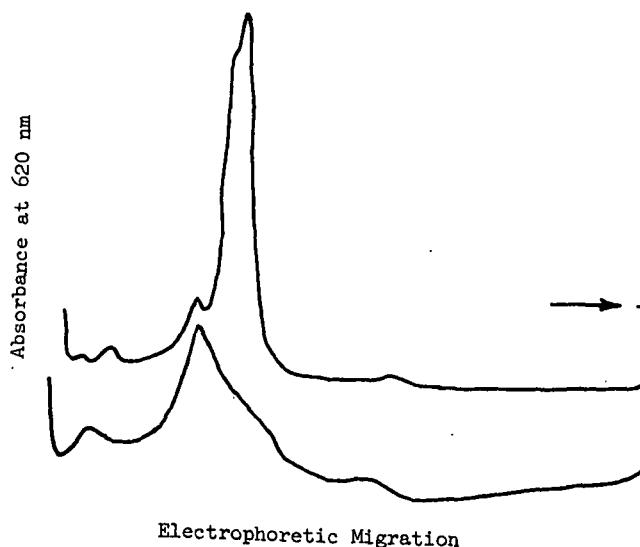




Figure 18. Anionic Electrophoretic Distribution Patterns of Peroxidase Extracted from Douglas-fir Hypocotyls and Roots and Assayed in the Presence (top) and Absence (bottom) of Added Hydrogen Peroxide

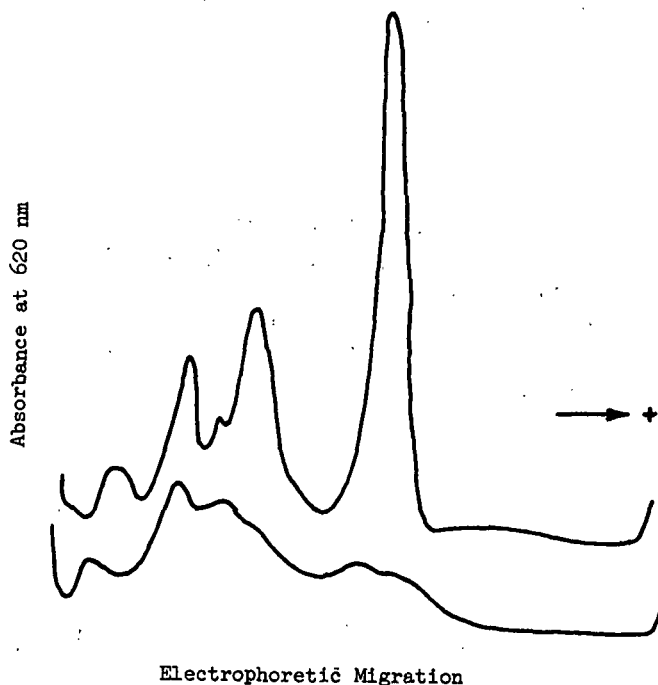


Figure 19. Anionic Electrophoretic Distribution Patterns of IAA Oxidase Extracted from Douglas-fir Needles and Assayed in the Presence (top) and Absence (bottom) of Added Hydrogen Peroxide

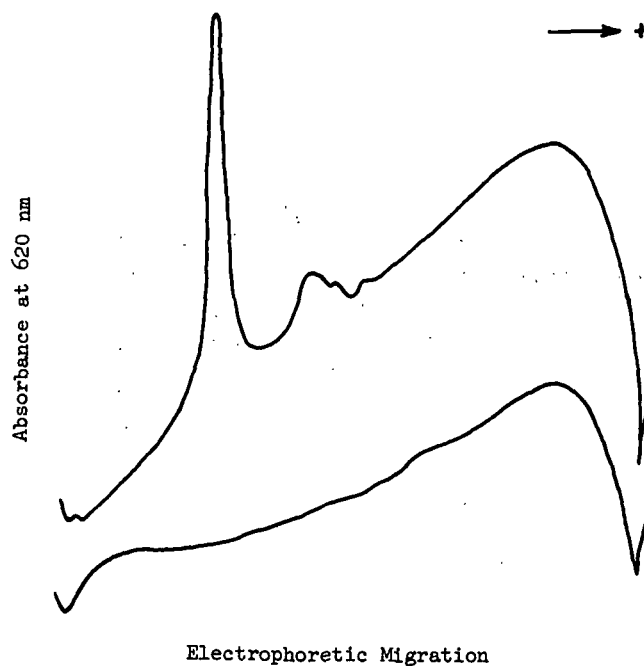


Figure 20. Anionic Electrophoretic Distribution Patterns of IAA Oxidase Extracted from Douglas-fir Stems and Assayed in the Presence (top) and Absence (bottom) of Added Hydrogen Peroxide

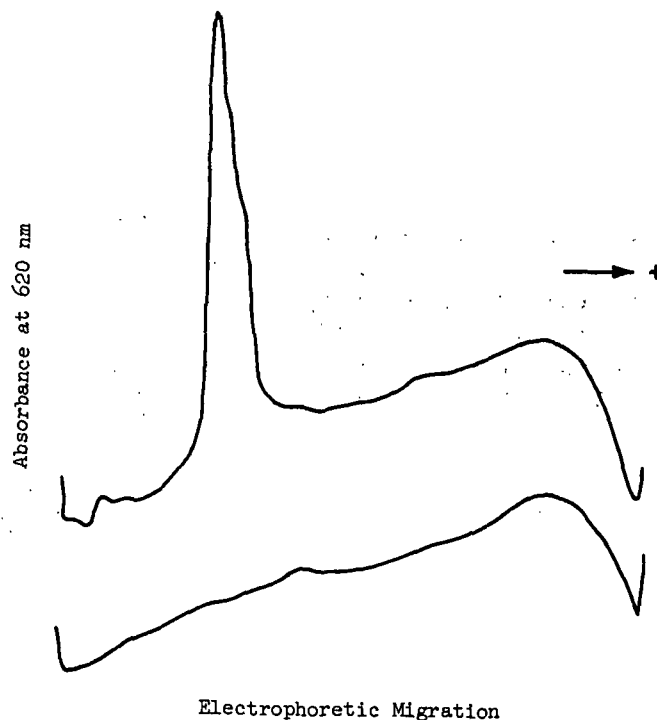


Figure 21. Anionic Electrophoretic Distribution Patterns of IAA Oxidase Extracted from Douglas-fir Hypocotyls and Roots and Assayed in the Presence (top) and Absence (bottom) of Added Hydrogen Peroxide

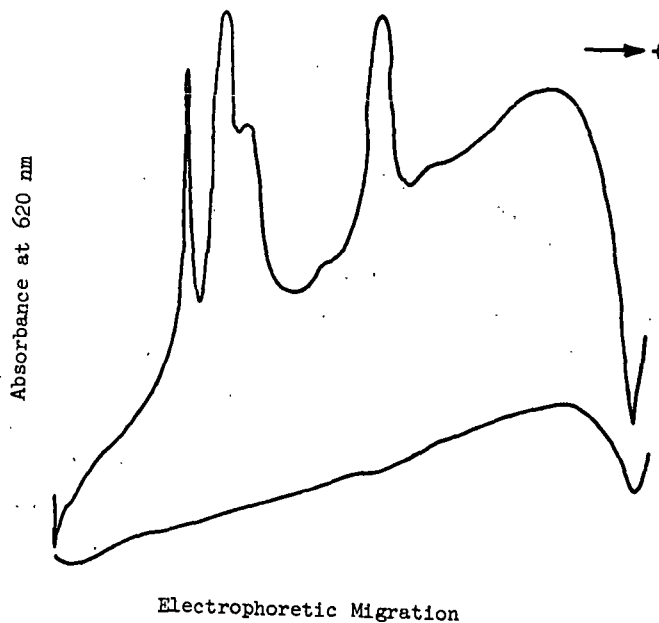
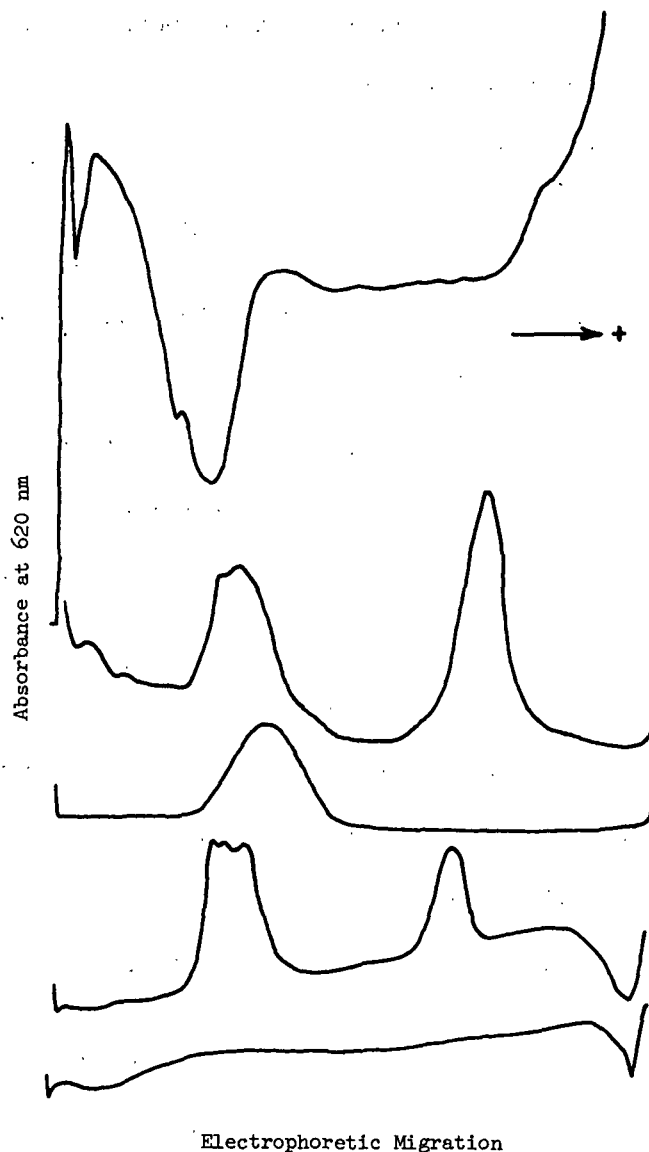


Figure 22. Anionic Electrophoretic Distribution Patterns of Catalase, Peroxidase, and IAA Oxidase Extracted from Douglas-fir Needles Callus. Top to Bottom: Catalase (Negative Peak), Peroxidase, Peroxidase Without Added Hydrogen Peroxide, IAA Oxidase, IAA Oxidase Without Added Hydrogen Peroxide



The relevance of the foregoing observations to embryogenesis is that small amounts of hydrogen peroxide were found to promote embryoid formation in suspension cultures although the effect was quite erratic. However, it has also been noted that callus extracts are characterized by substantial catalase activity (Fig. 22) which has the potential of keeping endogenous peroxide concentrations at a low level. As noted below, in vitro analyses of peroxide content of Douglas-fir seedlings reveals about 300 nanomoles per gram fresh weight while Douglas-fir callus tissues contain only about one quarter that amount on the same basis. The

use of thiourea to "protect" endogenous peroxide has also promoted embryoid formation and more reliably than peroxide addition, though still far from being the perfect answer. While this compound is a known catalase inhibitor (12), this is not necessarily its major effect in vivo.

An intriguing and very important aspect of this research to promote embryoid formation in suspension cultures is the general biological response to IAA oxidase promoters and inhibitors. It was noted above that hydrogen peroxide is a known IAA oxidase promoter and also occasionally stimulates embryoid formation. It has been found that other known IAA oxidase promoters such as p-coumaric acid (11) also promote embryoid formation and do it consistently. On the other hand, IAA oxidase inhibitors like catechol (13) do not promote embryoid formation and are, in fact, deadly at rather low concentrations. (See also section on Phenolics under Newly Initiated Studies.) From the evidence currently available it appears that a deficiency of IAA oxidase cofactors may be an important barrier to embryogenesis from conifer suspension cultures.

Comparisons of anionic isozyme patterns for peroxidase and IAA oxidase can also be made for loblolly pine. Compare the IAA oxidase patterns in Fig. 23 and 24 with counterpart isoperoxidase patterns in Fig. 7 and 9. Again one sees a similar picture developing as for Douglas-fir, and again no IAA oxidase was evident without added peroxide (not shown). Also, some of the isoperoxidases remain active in organized loblolly pine tissue without added hydrogen peroxide, but such activity is weak to nearly absent in the two loblolly callus tissue extracts (not shown). The isozyme patterns for loblolly pine stem callus (Fig. 7 and 23) resemble those of shoot-producing Douglas-fir needle callus (Fig. 15) which may be notable considering the weak activity in Fig. 9 and absent activity in Fig. 24 for stems. As stated before in the case of isoperoxidases, IAA oxidase patterns are not yet

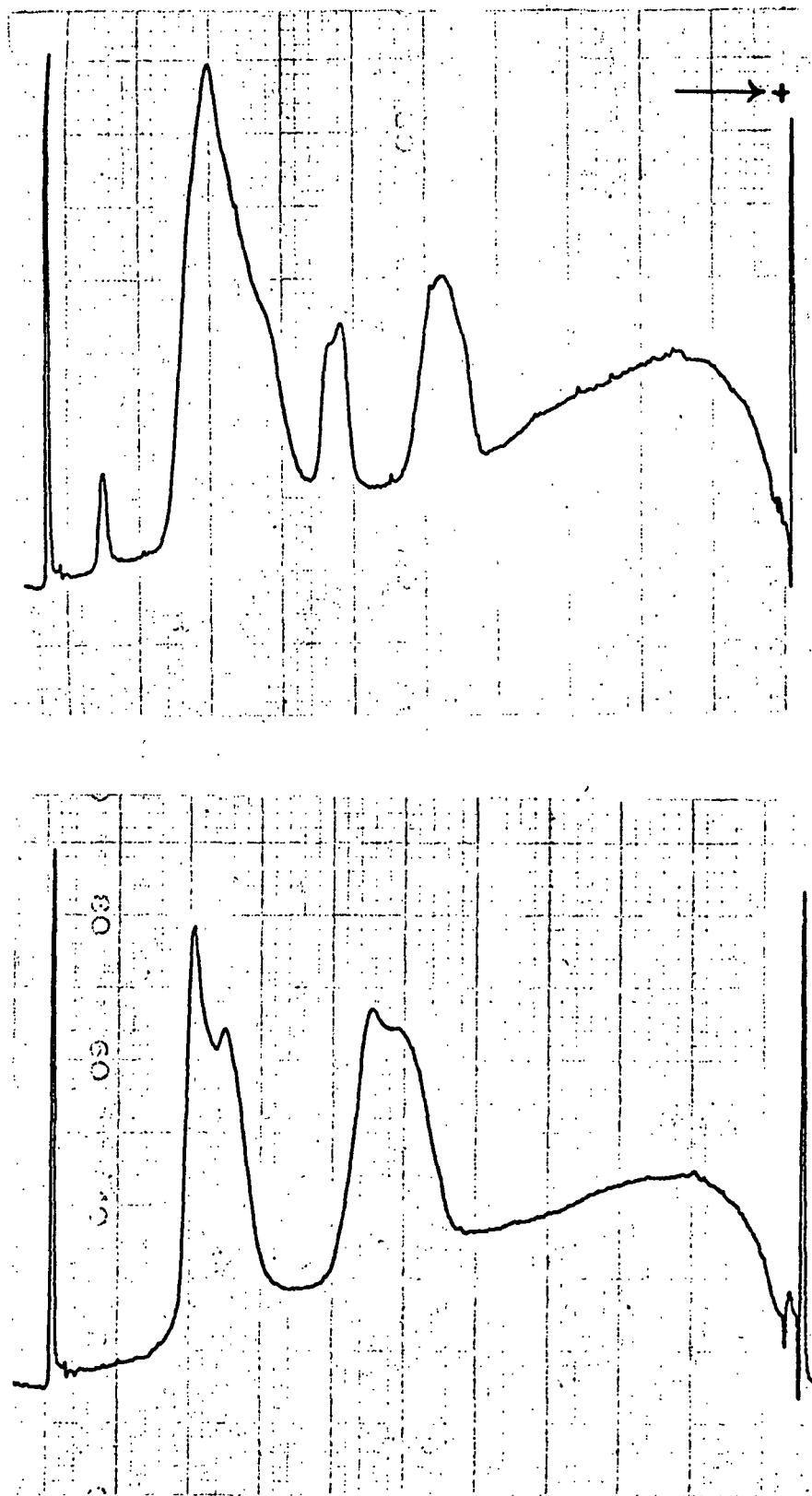


Figure 23. Anionic Electrophoresis of Extracts from Loblolly Pine Callus.  
IAA Oxidase Activity. Top: Cotyledon Callus. Bottom: Stem Callus

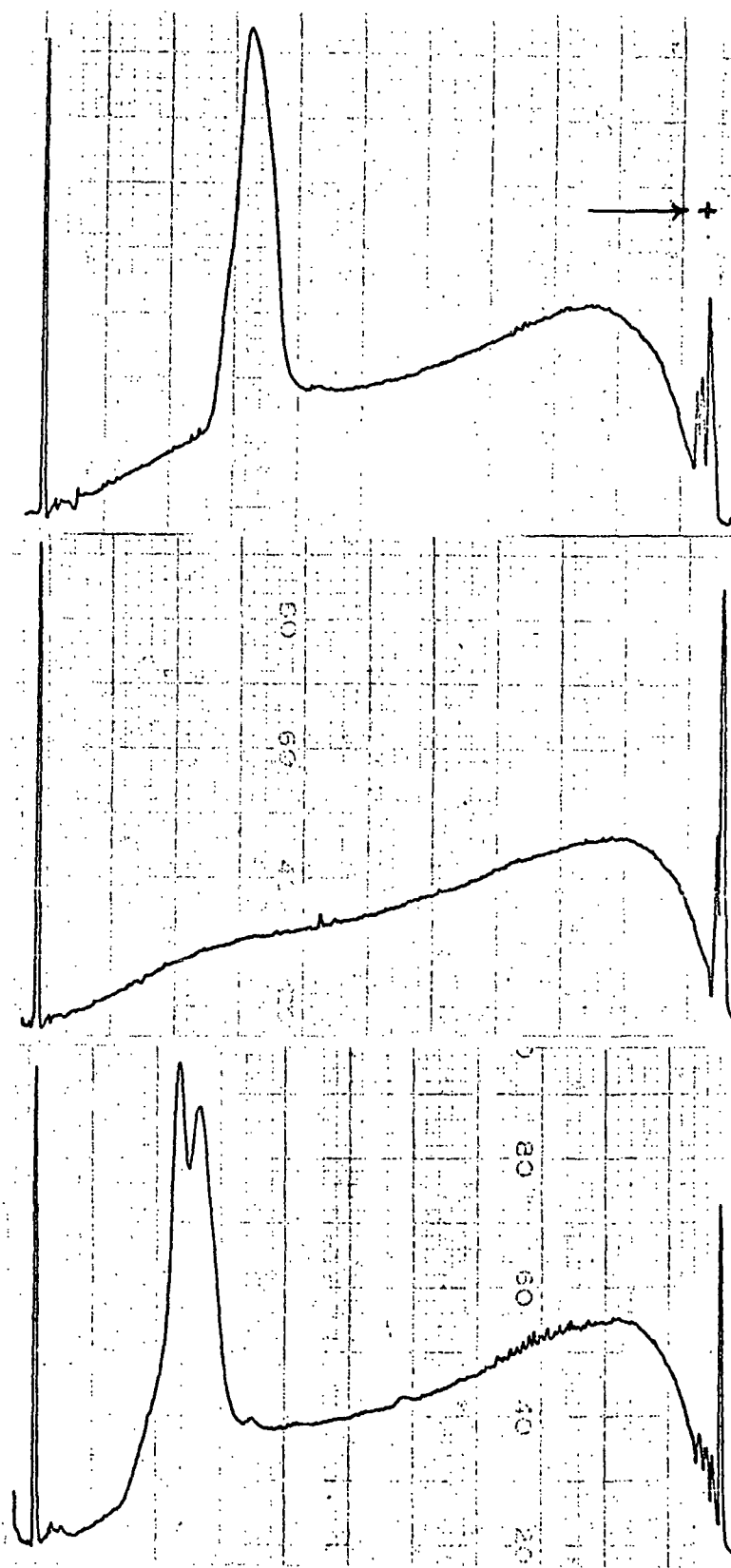


Figure 24. Anionic Electrophoresis of Extracts from Loblolly Pine Seedling Tissues. IAA Oxidase Activity. Top to Bottom: Needles, Stems, Hypocotyls-Roots

available for loblolly needle callus extracts. Also, with that exception, cationic electrophoretic separations of IAA oxidase isozymes for all other Douglas-fir and loblolly pine tissues normally considered here are available. Only one example will be given however. Compare the cationic IAA oxidase distribution for the loblolly callus extracts in Fig. 25 with the cationic isoperoxidases in Fig. 8. Generally, the cationic patterns are not receiving much attention at the present time because the anionic patterns appear relevant, are often better-defined, and are more readily related to the literature. However, there are also some differences in cationic patterns that may be exploited in the future. For example, notice that the loblolly stem callus extract in Fig. 25 shows no IAA oxidase activity while the cotyledon callus extract does. Peroxidatic enzyme activity released into suspension medium is also accompanied by IAA oxidase (Fig. 26) although clonal differences have been found.

IAA oxidase activity from both loblolly pine and Douglas-fir tissues appears to be dependent upon peroxide regardless of which organized or unorganized tissue is under consideration. Although a strong relationship is evident between isozymes of IAA oxidase and peroxidase, not all isoperoxidases share in the peroxide requirement. Therefore, it would seem that knowledge of the availability of peroxide and other IAA oxidase cofactors and regulators in given tissue types as determining factors for endogenous auxin levels might be useful.

#### Plans

With the exception of some noted small gaps in the enzyme data for loblolly pine that need closing, attention is now turning to IAA oxidase and its role in morphogenesis. Quantitative data on this activity will be sought in various tissues and correlated with cofactor and inhibitor availability. The response of the different IAA oxidase isozymes to various cofactors and inhibitors will

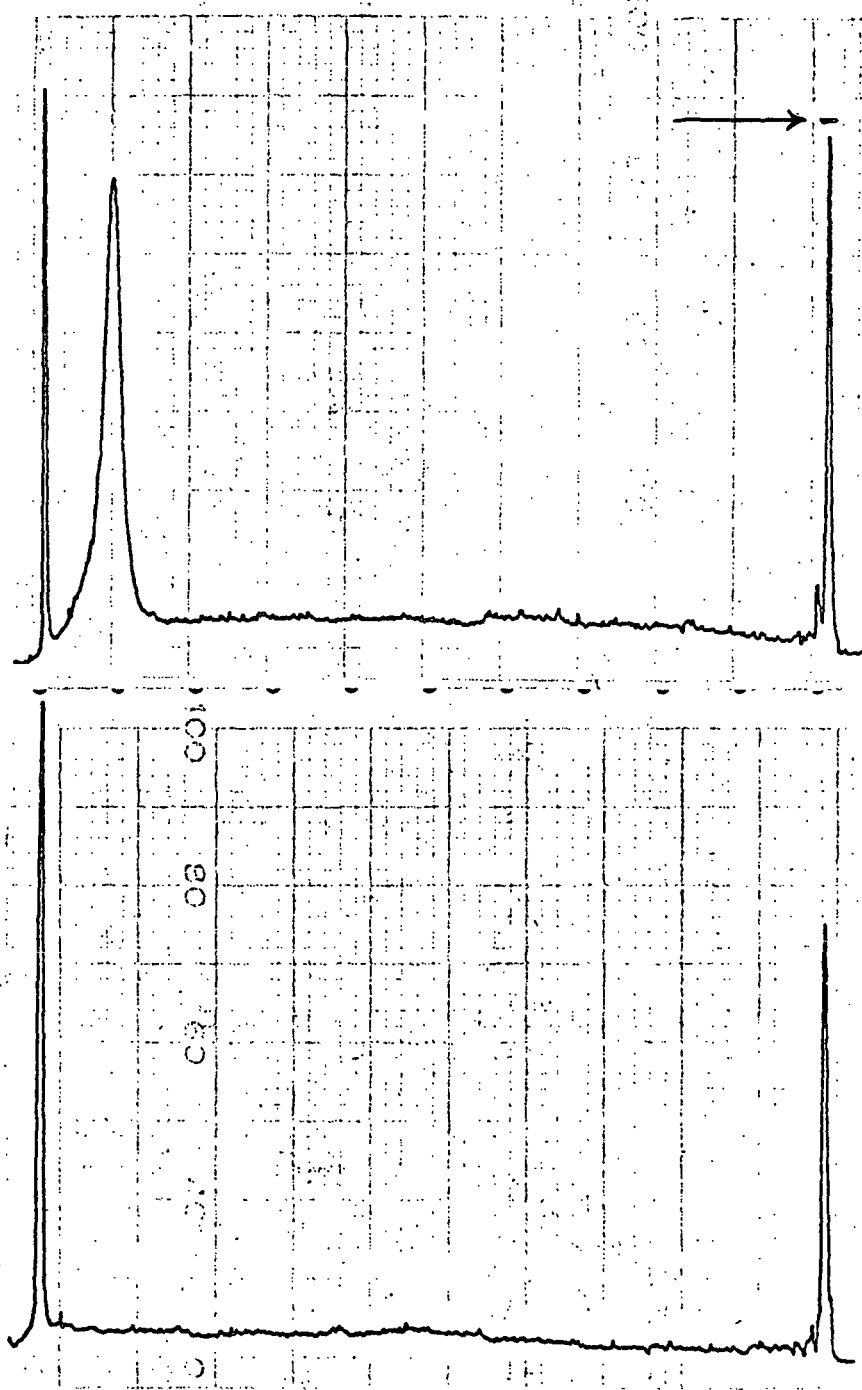


Figure 25. Cationic Electrophoresis of Extracts from Loblolly Pine Callus. IAA Oxidase Activity. Top: Cotyledon Callus. Bottom: Stem Callus



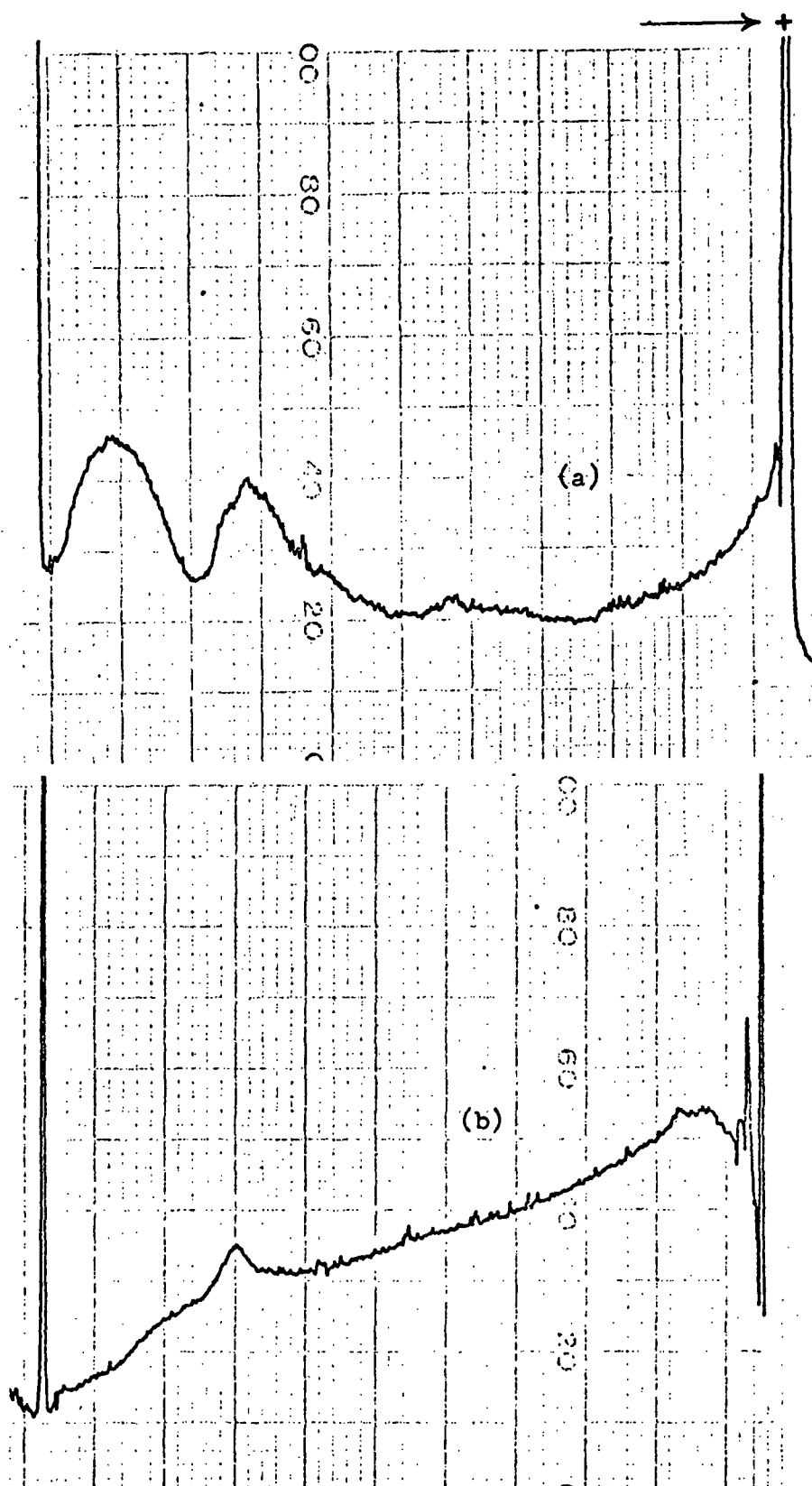


Figure 26. Anionic Electrophoresis of Cell-free Suspension Medium After Culture of Douglas-fir Stem Callus Cells. Peroxidase Activity (a) and IAA Oxidase Activity (b)

be examined. Eventually, it may become necessary to separate the isozymes in order to characterize them in this manner. At least in the near future the other enzymes, such as catalase, peroxidase, phenol oxidase, and superoxide dismutase will be studied only in so far as they relate to IAA oxidase.

## PEROXIDE ANALYSES

### Introduction

Assessment of the validity of the peroxide regulation hypothesis requires some quantitative data on peroxides if they exist in tissues of developmental interest. Peroxide analyses of various tissues were conducted to see if there might be parallels, for example, between distributions of catalase and other peroxidases and peroxide content. Results reported here are from in vitro assays and are intended to help assess the validity of the peroxide regulation hypothesis. The overall relationship between this work and other investigations is presented in Appendix Table VI.

### Methods

Peroxide extraction and analysis was adapted from Sagisaka (14). Tissue was ground in the cold room in 5% trichloroacetic acid (40 ml/g dry wt.) with washed sand (2 g/g dry wt.). Samples were assayed at least in triplicate accompanied by a reagent blank control. Since the color developed in the assay was not completely stable, the mechanics of the technique allowed better data generation when absorbances of samples and controls were separately recorded for 3 minutes versus a water reference rather than against each other; resultant time progress curves were then extrapolated to zero time, and the control values were used to correct sample values. It should be cautioned that this procedure, as used here, does not distinguish hydrogen peroxide from other peroxides. Peroxide recovery in extraction was about 85%.

## Results

Table II presents some typical data for Douglas-fir and loblolly pine tissue extracts. All seedling and callus tissues examined so far have substantial peroxide content. Since these are in vitro analyses, there remains some question as to how representative these data are of the in vivo situation. An in vivo assay for peroxide is contemplated which may permit us to speak to this point. Also, analyses of different seedling parts have not been run separately at this time. There is further the matter of how to express these results to draw meaningful conclusions; values in the table are given on both fresh weight and dry weight bases, but some other basis like protein might be used. The fresh weight basis suggests that the concentration of peroxide is low in callus tissues relative to seedling tissues whereas the dry weight of seedlings is largely fiber which causes problems in use of the dry weight basis for comparison.

Values for light- and dark-grown loblolly pine tissue appear in the table. Dark-grown Douglas-fir was not available at the time this investigation was conducted. It was thought that a low peroxide content in dark-grown tissues might be indicative that photorespiration was a major peroxide generator. While photorespiration may be contributing to the peroxide found in light-grown callus extracts, results now available cannot be used in support of that idea but rather suggest that other more significant peroxide generation occurs. However, glycolic acid, the primary substrate of photorespiration, has been used in feedback research with Douglas-fir suspension cultures (see discussion in Feedback Research).

It was noted elsewhere above that suspension cultures of Douglas-fir release peroxidases to the growth medium. Peroxide analysis shows that the peroxidase is accompanied by some peroxide, but, as might be expected, the amounts found are quite variable if enzymes (including catalase and peroxidase) are not

inactivated. Although more data are needed here, it appears also that Medium 26-10 gives a higher control value than just water when used in a reagent blank.

TABLE II  
PEROXIDE CONTENT OF TISSUES

Tissue	Sample Replicates	Nanomoles Peroxide Corrected for Control	
		Per g Fresh Wt.	Per g Dry Wt.
Douglas-fir seedlings	1	299	786
	2	296	780
	3	316	830
	4	308	810
	5	306	806
Douglas-fir stem callus	1	65	1100
	2	81	1370
	3	106	1786
Douglas-fir cotyledon callus nonshoot producing	1	66	1095
	2	77	1267
	3	96	1575
Douglas-fir cotyledon callus shoot producing	1	75	1227
	2	83	1370
	3	77	1271
Loblolly pine stem callus light-grown	1	21	351
	2	22	366
	3	22	372
Loblolly pine stem callus dark-grown	1	41	689
	2	41	675
	3	40	660

In all of these peroxide analyses, which are based upon iron oxidation, controls without extract show a feeble increase in absorbance with time after KCNS addition. Some samples exhibit the opposite trend, particularly Douglas-fir stem callus extracts. Such samples appear to contain labile unknown factors (reducing agents?) that lead to destruction of the ferrithiocyanate complex. It

is also apparent in the table that replication in the case of Douglas-fir stem callus extracts is rather poor. Here, as in other assays with this tissue, it has been observed that, despite timed reagent addition, each succeeding sample analyzed has a higher peroxide content as do deliberately aged extracts. It is thought that the high water content of the tissue dilutes the trichloroacetic acid to the point where it is no longer strong enough to stop enzyme activity. This probability is under investigation.

The tentative conclusion drawn from the data presently available is that the peroxide concentration in callus tissue is low relative to levels in organized tissues. However, this in vitro extrapolation needs confirmation from in vivo analyses. At the present time the results are consistent with a possible failure of unorganized tissues to meet a peroxide requirement for IAA oxidase activity.

#### Plans

More in vitro data on the peroxide concentrations in needle callus and needles of both Douglas-fir and loblolly pine will be obtained. Since extrapolation of such values to the in vivo situation is rather tenuous, attempts will be made to obtain some direct estimations of peroxide concentrations in vivo. Prospects for this in unorganized tissues are reasonably bright, but organized tissues are more apt to pose a serious technical difficulty.

#### ELEMENTAL (METAL) ANALYSES

##### Introduction

These analyses of callus and seedling tissues were conducted as an aid to medium development, i.e., to provide a basis for feedback regarding beneficial qualitative and/or quantitative changes that might be made in components common

to most media. These components are mostly metal cations but do include some other elements, such as P, detectable by emission spectroscopy. If these analyses continue to prove useful, similar data will be acquired for loblolly pine. The tissue analyses also led to a further analysis of agar as detailed below.

#### Methods

These analyses were conducted by the Analytical Department at the Institute using the arc emission spectrography technique with tin as an internal standard.

#### Results

Elemental analyses for Douglas-fir tissues given in Table III include results for seedlings, needle callus, and four samples of stem callus. Several elements appear to deserve some further attention, but particularly striking is the accumulation of K and Na. It could be quite important to single out the Na for immediate attention since only a trace of Na is provided in the media used. Our search for the Na source led to the agar supply as the most likely culprit although the  $\text{Na}_2\text{EDTA}$  used to chelate Fe brings in a small amount of Na. Elemental analysis shows common agar to be Na rich with at least seven times more Na than any other element revealed by emission spectrography. Experiments have been underway for some time now to ascertain the effects of reducing the Na supply to cultured cells. It may be important to do this beginning at the stage of callus initiation. However, since Na and K are active transport species, the problem (if it is one) may lie deeper than simple availability. Results to date show only that there are effects on callus friability. Some quantitative medium adjustments may be anticipated for other elements in light of these analyses.

TABLE III  
ELEMENTAL ANALYSES OF DOUGLAS-FIR BY EMISSION SPECTROSCOPY

Element	Ovendry Weight, %					
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Ash	5.3	6.0	4.6	8.0	4.7	10.9
Ba	0.0029	<0.0005	<0.0004	<0.0006	<0.0005	<0.0009
B	0.0052	0.0049	0.0048	0.0076	0.0050	0.0094
Si	0.32	0.010	0.0088	0.016	0.010	0.022
P	0.14	0.32	0.33	0.39	0.30	0.40
Mn	0.0052	0.012	0.013	0.014	0.0076	0.019
Al	0.079	0.0004	0.0003	0.0008	0.0005	0.0010
Fe	0.069	0.032	0.030	0.056	0.025	0.059
Mg	0.30	0.10	0.090	0.14	0.082	0.24
Pb	0.0003	<0.0001	<0.0001	<0.0002	<0.0001	<0.0002
V	0.0010	<0.00006	<0.00005	<0.00009	<0.00006	<0.00012
Ca	0.68	0.24	0.20	0.34	0.18	0.42
Mo	<0.0001	0.0009	0.0010	0.0013	0.0008	0.0012
Zn	<0.0086	0.018	0.020	0.030	0.020	0.030
Na	0.11	0.40	0.28	1.1	0.49	1.2
Cu	0.0012	0.0003	0.0003	0.0015	0.0014	0.0005
Ti	0.0053	<0.0002	<0.0002	<0.0003	<0.0002	<0.0004
K	0.72	2.1	1.4	2.8	1.6	3.7

Co and Cd were also sought but were undetectable in any of the tissues.

Sample 1 = Douglas-fir seedling.

Sample 2 = Douglas-fir needle callus - 5 subcultures on Medium 10.

Sample 3 = Douglas-fir stem callus - 17 subcultures on Medium 10.

Sample 4 = Douglas-fir stem callus - 68 subcultures on Medium 10.

Sample 5 = Douglas-fir stem callus - 68 subcultures (41 on Medium 3 then 27 on Medium 17-8).

Sample 6 = Douglas-fir stem callus - 67 subcultures on Medium 17-8.

## GROWTH AND ORGANIZATION IN SUSPENSION CULTURES

### EFFECTS OF CHEMICAL AND ENVIRONMENTAL FACTORS

#### Introduction

Listed in Part I of the Research Program (Appendix Table VI) are studies involving autoclaving, cell cluster size, and sucrose level. The first two factors will be discussed separately in following sections and the sucrose level will be discussed here as one of several factors we have been trying to optimize. We are also interested in the quality and quantity of light and its duration, as well as in drum speed of revolution for suspension cultures, test tubes versus flasks for suspension vessels, the effects of 3-5 day intervals between changes of medium versus not changing the medium, effects of a cold pretreatment of callus before being used for suspensions, and the effects of a commercial fermentation product. These, along with sterilization and cell cluster size constitute the main empirical studies carried out in the tissue culture lab. The other half of our work is concerned with feedback experiments in cooperation with the biochemistry lab. Some of the studies have not been completed, so a general approach will be adopted to report our progress.

Empirical studies are used to adapt the best results of the feedback studies to the optimum environmental factors available in the laboratory. Thus, a significantly new level of embryoid induction, stimulated by a feedback factor, would immediately call for an experiment using the best feedback treatment, coupled with the best alternatives of the environment determined by empirical studies. This is a continuous process, and is complementary to the biochemical feedback program. For each factor, a separate short section will be used instead of one section of Results and Discussion. Only the general suspension method will be outlined in the Methods and Materials Section.



The purpose of the sections that follow is to update the reader regarding the status of research underway on sucrose level; light quality, quantity and duration; type of experimental vessels; frequency of media change; and the use of a commercial fermentation additive.

#### Methods and Materials

From the start of this project until this past spring, we have used the old method of inocula preparation as well as basic Medium 26-10 as a starting point for cell suspension research. The new method will be presented later and is now routine. In both methods each treatment was replicated three times. Each replicate consisted of 10 ml of treatment solution in one 25 x 150 mm test tube with metal closure. To each tube was added about 0.25-0.5 cc of macerated callus tissue. (One of the main features of the new method is the screening of callus to remove large callus pieces.) Soft green callus was preferred because it dissociated easily when the tubes were rotated on a drum at 10-15 rpm, under fluorescent lights in the lab of 4000-5000 lx on for 16 hr per day at  $24 \pm 1^{\circ}\text{C}$ . (In the new method the drum is slowed to 1 rpm in lower light.) Suspensions were usually monitored 1-2 times a week by microscopic examination, and the cells were rated as to growth by the amount of green and new cells, and also the frequency and size of embryoids was noted. New suspensions were made fresh for each experiment, and each tube received fresh medium generally once a week. Occasional long-term cultures were left without changing for up to 50-80 days.

#### Sucrose Level

Sucrose levels of 2-4% are preferred by most plant cultures, and we normally use 3% in agar Medium 3 and 10, and use 3.5% in liquid Medium 26-10. We ran one experiment using 2, 3, 3.5, and 4% sucrose in Medium 26-10 in suspensions. Embryoids were observed in about equal numbers in media with 3-4% sucrose,

which were all better than with 2%, possibly with slightly greener cells and slightly more embryoids at 3.5% sucrose. However, we were not able to quantify significant differences among 3, 3.5, and 4%. So, until we can observe differences, we plan to continue to use 3.5% sucrose. We have not examined sucrose levels to the extent we would like, because we are waiting until we have a fairly satisfactory embryoid-initiation medium that will give consistently high frequencies of embryoids. However, in new studies just started with developer media, we are testing the effect of reduced sucrose levels as a factor for enlarging embryoids. This will be discussed later under new studies.

#### Light Quality and Quantity

##### Quality

In the past we have used Cool White fluorescent lights, and have obtained embryoids from cells in suspension cultures as well as shoots from callus cultures. However, after tests using the lavender Sho-Grow fluorescent bulbs, cells appeared greener in suspensions. This may be due to the higher red light than in Cool White fluorescents. Recent work also indicates that lavender light, without the usual blue light of most fluorescent lights, is beneficial for organogenesis in Douglas-fir cultures. Currently we are testing Sho-Grow lights alone, but regular CW lights are also used in the lab during daytime working hours. We may place drums in an incubator to control all the light.

##### Quantity

We have grown suspensions in light at 1,000-8,000 lx, and thought that we got faster response to feedback additives (embryoid initiation) in about 4,000 lx and best embryoid development at lower levels of about 1,000 lx. We are trying to find one suitable combination of quality and quantity to get the maximum response in both feedback and embryoid development experiments. In recent tests we have

reduced the light to 100 ft-c or to darkness to compare embryogenesis in different amounts of low light intensity. We may find that the light requirements are different for embryogenesis than for development, so this study will continue along with testing other light factors. If we also decide to maintain continuous suspension cultures, a higher light level may be necessary than for either embryoid initiation or development.

#### Experimental Vessels

We routinely use tubes for suspension cultures. We can accommodate several hundred tubes among a few rotating drums in a relatively small space, and tubes appear to be satisfactory for feedback, and possibly also for embryoid development studies. In several experiments using 125 ml flasks, we have not found shake cultures to be superior to tube cultures, and plan to use flasks only occasionally to test for embryoid development along with tube controls. The flasks may be aerated better than tubes. However, the motion in flasks appears to be more random than in tubes, and possibly not as conducive to polarization motion that may or may not be necessary for embryoid initiation.

#### Change of Medium

In most experiments, especially of a preliminary nature, we usually make up the treatments once, run them for 1-2 weeks or longer, then discard them. For embryoid development, one change of fresh medium appears to be the best. However, frequent changes of medium at intervals of 3-7 days appears to keep the cells greener than when there is no change. Since there does not appear to be significant differences in growth among changes at 3, 5, or 7 days, we routinely make changes at 7 days. We have not yet explored why changing the medium keeps the cells greener. It could be simply the renewing of nutrients, the removing of wastes or inhibitors, or a combination of the two. However, frequent changes

also appear to remove a chemical factor that helps embryoids develop into larger embryoids. A simple addition of some new medium is better in these cases, but addition can only be done a few times during a long culture. In one recent experiment using feedback information, medium was changed in different treatments from once after three days, up to twice a week for four weeks. The most embryoids were initiated after two changes and after one additional week.

However, as our medium improves, we expect that the need to change may diminish if it is a problem of nutrition. On the other hand, if waste metabolites are building up in the systems, we may either have to reduce the buildup or remove the wastes by changing the medium 1-2 times a week.

#### Cold Pretreatment

We have tried pretreating callus and suspension cells with cold before placing them on a rotating drum, in attempts to "vernalize" the cells and trigger embryoid initiation. Usually a slight increase in the frequency of embryoid initiation is noted, but no significant change can be attributed to cold treatments. An exception is one experiment with callus cultures, where the callus receiving 3-5 days of cold (3°C) grew faster and greener and longer than did controls that did not receive cold. We have not followed up this approach because the better growth did not also increase embryoid initiation.

#### Fermentation Product

A commercial preparation was made available to us to test for embryoid induction in callus cultures as well as in suspension cultures. A strain of bacteria was cultured commercially (not by us), and the bacterium, substrate and products were all sterilized together and packaged for sale as a stimulator of plant growth. We made dilutions of the original thick material from  $10^0$  to  $10^{-8}$

in liquid Medium 26-10 for suspensions, and also dropped the same solutions onto callus still in Petri dishes.

The undiluted and the 10-fold dilution killed the callus and most cells in suspensions. However, at  $10^{-5}$  and  $10^{-6}$ , there was increased embryoid initiation over the controls. Unfortunately, we do not at this time believe that the increase in embryoid initiation was superior to some other known additives we have tested, and at present we have discontinued testing this material.

#### Future Plans

We will continue to test various physiological and environmental factors for suspension culture. These include pH, light quality and a preliminary scale-up for the mass production of cells. If we are able to induce high frequencies of embryoids in an initiator medium, and under conditions that are different than required for development into embryos, we will then optimize each set of factors. The plan is to have one set of conditions for growth and initiation and another for development. Chances are small that one universal environment or medium will serve all purposes.

#### STERILIZATION

##### Introduction

Nutrient media may be sterilized by autoclaving, microfiltration, or by adding an antibiotic agent to reduce loss by contamination. Autoclaving is the most convenient and cheapest, but the high heat and pressure necessary to kill all bacterial and fungal microorganisms also degrades vitamins, some growth hormones, a few amino acids and sucrose. For most routine culturing, however, autoclaving for 15-20 min at  $120^{\circ}\text{C}$  and 15 psi is standard practice. Microfiltration offers a method of sterilization without heat through pores ranging from 0.2-0.5  $\mu\text{m}$ ,

but the method is relatively expensive and if any leakage occurs, contamination is not readily apparent. The addition of antibiotics has tended to inhibit the growth of plant cells and is not usually practiced among plant tissue culturists.

One recent view is that some sucrose degradation may be beneficial to cell growth and differentiation, but too much degradation is inhibitory. This approach invokes the possibility that optimum degradation requires insufficient heat to kill all contaminants, requiring additional sterilization by filtration.

The purpose of this study was to determine the effects of autoclaving or filtration, or a combination of the two on cell growth and embryoid initiation in Douglas-fir cell suspension cultures. This section reports the general results of this study.

#### Methods and Materials

Most autoclaving was done in a Market Forge Sterilmatic, which heats its own water to produce steam. Some comparative experiments were run in an American Sterilizer in another lab, which uses outside steam. For most experiments we used Medium 26-10 liquid medium, testing the following factors:

Cold start vs. preheating

Dial setting vs. actual time of each part of the cycle (up to full heat and pressure, at full heat and pressure, exhaust to zero pressure)

Volume of liquid vs. size of flask holding medium

Autoclaving vs. cold filtration vs. autoclaving plus filtration

Cold-filtered control treatments were run with most autoclaved treatments, using two different pore sizes of 0.2 or 0.45  $\mu$ m, in filter holders 25 or 47 mm in diameter. Large, 100 ml filter holders were also tested using the large-pore filters. In some experiments, autoclaving was followed by filtering of some of each treatment.

After the media were treated, a small amount (1-5 ml) was poured directly onto the surface of agar Medium 10. If bacteria were present they grew in colonies and were verified by microscopic examination. In some experiments Bacillus megaterium was added to the medium before sterilization. Each treatment was also used for suspension cultures with Douglas-fir callus tissue. Subsequent cell growth and embryoid initiation were monitored, along with possible contamination.

### Results and Discussion

The most significant result was that the total time of the routine autoclaving cycle was reduced 41% by making two changes in the operation procedure. One was to preheat the autoclave for five minutes before adding the medium for sterilization. The second was made during exhausting, immediately after sterilization, where normal slow exhausting was continued from 15 psi down to 10 psi, then the selection lever was flipped to fast exhaust. This allowed enough pressure to escape during slow exhaust so that the liquid would not boil out during fast exhaust. These two innovations significantly reduced the total time of exposure to heat by the medium, by reducing both the pre- and the poststerilization times of the autoclaving cycle. This permitted the selection of a specific time of sterilization sufficient to kill all contaminants, while still reducing total degradation time of exposure to heat. The total times of cycles in the American Sterilizer were about 20% less than in the Market Forge. However, comparative tests were designed so that the killing times at full heat and pressure were the same in both autoclaves. Effectiveness of killing and effects on cell growth and differentiation were about the same for both autoclaves.

A dial setting of 8-9 (total time of 12-13 min) on the Market Forge was the minimum for complete sterilization by autoclaving, using volumes of 500 ml of medium. However, smaller volumes usually were not sterilized, possibly because

of the smaller residual heat carried by the lower volumes. Cell growth and embryoid initiation was about the same whether the medium was autoclaved for the minimum time, sterilized by filtration alone, or autoclaved for five minutes followed by filtering. However, in some experiments filtration alone or a combination seemed to be better than autoclaving alone. Also, significant differences were not apparent whether the autoclave dial was set at 8-16 min.

### Conclusions

Based on our investigations, it appears that filtering or a combination of autoclaving and filtering has about the same effect on growth and embryogenesis as autoclaving alone. We have, therefore, discontinued this study.

### Future Plans

For routine suspension media we will sterilize by autoclaving at a dial setting of 16 min, using the two improvements of preheating and slow/fast exhausting. For feedback experiments we will continue to sterilize by filtration through 0.45  $\mu\text{m}$  pores. At such a time when we develop reliable initiator-developer media, we may then repeat critical parts of this study to determine if there are significant differences in the three methods of sterilization tested.

### CELL CLUSTER SIZE

### Introduction

In several laboratories around the world, embryogenesis is routinely initiated in suspension cultures of carrot callus. In one lab, one of the steps of inocula preparation is screening or sieving callus cells as a slurry through stainless-steel sieves with openings ranging from 45-75  $\mu\text{m}$ . Cell clusters in the 45-75  $\mu\text{m}$  fraction usually contain small embryoids preformed in callus, and are placed in suspension cultures for development into embryos and plantlets. Cell



clusters smaller than 45  $\mu$ m usually do not have preformed embryoids, but often they are initiated de novo in suspension cultures from mostly single cells and a small percentage of clusters with 2-5 cells (15).

In other labs, optimum cell-cluster size varies, and most workers now use woven-nylon material for sieving. However, the approach seems to be similar and involves using single cells or small cell clusters for embryogenesis. One implied reason why this works may be that large cell clusters exert an inhibiting effect on embryogenesis, possibly by the accumulation of phenolics in the large clusters. The purpose of this study was to investigate the effects of different cell-cluster sizes on embryogenesis in Douglas-fir suspensions. (The problem of possible phenolic inhibition is being investigated in a separate study and will be presented later in this report.)

This section reports techniques developed for isolating specific cell fractions and preliminary results on the effects of cell-cluster size on embryogenesis in Douglas-fir suspension cultures.

#### Methods and Materials

Several types of sieves have been designed or adapted at IPC. When Dr. Richard Smeltzer was a student here, he designed a series of wire-screen inserts that could be placed inside a test tube and used to sieve cell suspensions through openings 150 and 200  $\mu$ m in size. This gave good uniform suspensions of clusters averaging 1-2 dozen cells each. However, the volume of the tube sieves was low and later we suspected possible metal poisoning from the copper wires, so this method was discontinued.

In answer to an inquiry, Dr. Fujimura, University of Tokyo, sent samples of woven nylon with 77 and 161  $\mu$ m openings, along with details on how to construct

sieves from small crucibles. However, instead of grinding off the bottom of crucibles, we knocked out the bottom of Selas filter crucibles and cemented nylon mesh of different sizes over the openings. At various times we tested sieves with openings from 37-1000  $\mu\text{m}$ . Two disadvantages readily became apparent, the low capacity and inability to autoclave the epoxy without softening. We sterilized with 95% ethanol and also made larger sieves.

To get more capacity, plastic 250-ml bottles were sawn across about 1 cm below the shoulder and again at the bottom. A piece of nylon mesh was cemented between the rejoined bottle at the shoulder, and a series of sieves was made with openings from 37-500  $\mu\text{m}$ .

The third method of separating cell-cluster sizes was not by filtering but by differential decanting of callus slurries after settling for different times. With practice and microscopic monitoring, it was possible to separate cell clusters into three fractions, roughly corresponding to those recovered by sieving using 37-125, 125-250 and 250-500  $\mu\text{m}$  openings.

Douglas-fir callus tissue was macerated with forceps and dropped into liquid medium and usually shaken in flasks for 1 hr at 100-120 rpm. Normally one dish of callus was placed in one 125-ml flask in 30 ml of medium, but for the differential decanting larger volumes were used in larger flasks.

### Results and Discussion

Table IV shows the results of one sieving experiment using a Douglas-fir callus culture of average softness. Some of our new cultures are much softer and give better results, and the firmer ones are discarded or used for other studies. For the fractions of cell-cluster sizes we recovered, both volume and weight were reduced roughly by an order of ten for each fraction. The average number of cells

per cluster was about 3-8 in the 125-250  $\mu\text{m}$  fraction and about 2-3 in the 74-125  $\mu\text{m}$  fraction and included occasional single cells. In other experiments, a few single cells and occasional clusters of 2-3 cells were found in 37-45  $\mu\text{m}$  fractions, but the volume of cells recovered approximated 0.01% or 0.01 ml from one dish of callus three weeks after subculturing. So far this is rather poor efficiency for obtaining single-cell suspensions. Also, about one-third of the cells in the callus were lost during maceration. However, this loss appears to be much smaller in some of the softer cultures.

TABLE IV  
VOLUME AND WEIGHT OF DOUGLAS-FIR CALLUS CELLS IN SIEVED FRACTIONS<sup>a</sup>

Fraction, $\mu\text{m}$	Volume		Wet Weight		Dry Weight	
	ml	%	g	%	g	%
>500	5.9	59.00	2.33	46.70	0.21	0.90
250-500	2.2	22.00	0.66	13.23	0.08	12.12
125-250	0.8	8.00	0.26	5.21	0.03	11.50
74-125	0.1	1.00	0.04	0.80	0.005	12.50
37-74	0.05	0.50	--	--	--	--
<37	--	--	--	--	--	--
Difference	0.95 <sup>b</sup>	9.50	1.70 <sup>c</sup>	34.06	--	--
Total	10.0	100.00	4.99	100.00	--	--

<sup>a</sup>Average of duplicate samples. These figures also agree with a separate run with the same callus source with a wet weight of 5.30 g before maceration and 5.15 g after maceration but before adding liquid, for an immediate wet-weight loss of 0.15 g due to maceration and evaporation in a few minutes.

<sup>b</sup>The estimated difference in callus volume was probably due to air spaces between large pieces of callus. The smaller the cell clusters the closer they packed together.

<sup>c</sup>The estimated difference in wet weight was probably due to maceration of the soft callus tissue, rupturing nearly one-third of the cells. The filtrate passing through the 37  $\mu\text{m}$  sieve was greenish-yellow and contained a thick suspension of cell organelles. However, the debris was not measured because it would not reflect the weight or volume it represented as whole cells. This callus source represents the average softness, with some cultures slightly firmer and some softer with better yields of smaller cell clusters.

On the basis of many experiments with several media and different separate and combined fractions, preformed embryoids that were initiated in the callus were mainly in the 125-250  $\mu\text{m}$  fraction. The 74-125  $\mu\text{m}$  fraction, which has always been a high producer of embryoids, contained some small preformed embryoids. In a few cultures, all cells are apparently completely undifferentiated and preformed embryoids are never found. With these cultures we have started suspensions, and in several experiments embryoids were produced de novo from undifferentiated cells within 1-2 weeks. The highest frequencies of embryoid initiation were in the smaller cell fractions, usually with no embryogenesis occurring in larger fractions. This supports the hypothesis that smaller cluster sizes are more apt to undergo embryogenesis than larger ones, possibly because of inhibition from larger clusters. However, this hypothesis may only apply to conifer cells that are known to produce phenolics in abundance and may not apply to herbaceous plant species, such as carrot, which lack such a highly developed phenolic system.

### Conclusions

This study showed that Douglas-fir callus can be separated into fractions according to the size of cell clusters. Sieving does not appear to damage healthy cells, but can be used to separate small clusters with few or no embryoids from larger clusters containing small-medium-sized embryoids. Using callus without preformed embryoids, embryogenesis tends to occur among smaller clusters, but the best treatments that initiate embryoids in small clusters also enhance cell growth and some embryoid initiation in larger clusters. Differential decanting bypasses sieving, and may be beneficial for preparing large amounts of smaller clusters for experiments. It also involves less handling and leaching of cell materials, as well as offering fewer points of possible contamination. While we will be using different fractions of cell clusters in most future studies, this study of how to obtain clean fractions is closed.

### Future Plans

We have already incorporated many results of this study into routine methods of inocula preparation and other studies on embryogenesis. The low percentage recovery of small cell clusters is hindering large-scale experiments of embryogenesis using those fractions, and this must be overcome. Two possible solutions are now under study for increasing the number of small cell clusters and single cells. One approach involves using enzymes to either separate cells only or to produce protoplasts and recover single cells after wall regeneration. This has proven successful in a few herbaceous species. Another method to test will be to use growth hormones to decrease the cell-to-cell bonding.

### PREPARATION OF INOCULA

### Introduction

The major emphasis of our program is to determine the trigger mechanism of embryogenesis in conifer suspension cells. Therefore, biochemical feedback experiments are an important part of the total effort. The best criterion at present, to determine whether a feedback treatment is significant, is either the initiation of embryoids or the enlargement of preformed embryoids in suspensions. On the basis of the results presented in the previous section on cell cluster size, we should use only small clusters for feedback experiments in order to get the best response. However, we also found that even our softest Douglas-fir callus will not dissociate as well as we would like into small clusters and single cells. We then had to make a compromise between the efficient use of callus for inocula and the use of cluster sizes most responsive to embryoid initiation.

The purpose of this section is to report on the present state of development of inocula preparation for feedback experiments.

### Methods and Materials

In feedback experiments, Johnson and Winton agreed on chemical additives or environmental factors to test, based on their probable influence on metabolic pathways associated with triggering embryogenesis. The results were used by Johnson to test hypotheses explained elsewhere in this report.

Three test tubes (replicates) per treatment are used, with 1 ml or less of callus tissue, in 10 ml of liquid Medium 26-10 containing the feedback additive. In early experiments, soft Douglas-fir callus was macerated with forceps and dropped directly into the treatment medium. Tubes were then covered and rotated on drums at speeds of 10-15 rpm in 5000 lx fluorescent light.

After sieving experiments were started to reduce cell cluster sizes, different fractions of callus were used for inocula to determine the maximum cluster size that would still show embryoid initiation or development.

### Results and Discussion

After testing sieves ranging from 37-1000  $\mu\text{m}$ , we settled for using all cell clusters passing through a 500  $\mu\text{m}$  sieve. We still lost over half of the original callus as large pieces, even after an hour or more of swirling the callus in flask cultures on the shaker at 120 rpm. However, enough cell clusters were recovered to provide inocula for ten or more treatments per experiment, and small but discernible differences were detected between treatments and controls in low frequencies of embryoid initiation or development.

The routine method of inocula preparation is diagramed in Fig. 27. The smaller fractions are now used entirely for small empirical studies, where a few media are tested for embryoid initiation. For all feedback experiments, the less-

than-500  $\mu\text{m}$  fraction is used. We have also reduced drum speed to 1 rpm and the light to 1000 lx.

## PREPARATION OF INOCULA

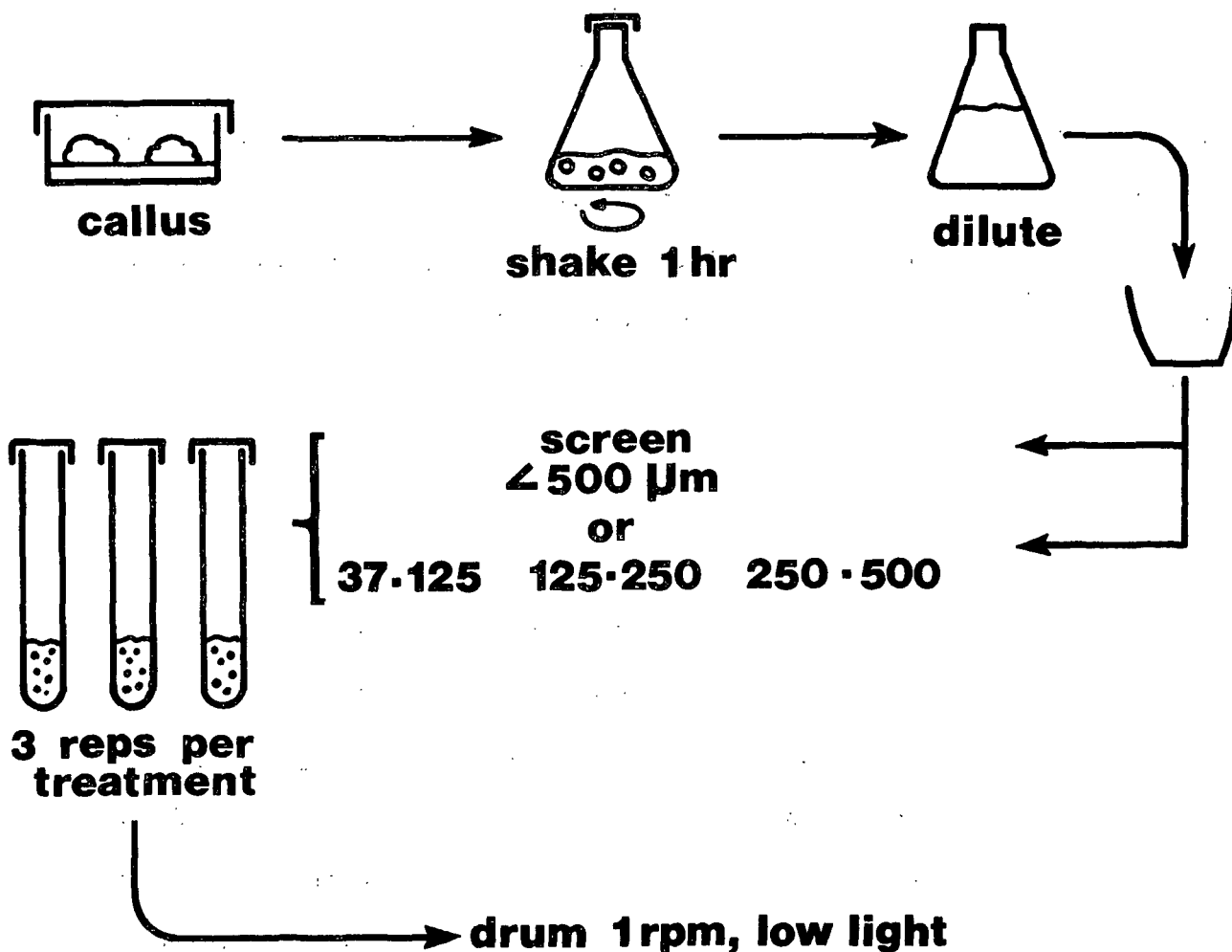


Figure 27. Flow Diagram for Preparation of Inocula

### Conclusions

The partial reduction of cell cluster sizes has greatly reduced variability in feedback experiments, in both cell growth and embryogenesis. This is a compromise between callus use and embryoid initiation, and does not represent the optimum or even a satisfactory stage of development. The next step is to further reduce the size of cell clusters by hormonal or enzymatic treatments, to

either obtain single cells or clusters of 1-3 cells, or perhaps going to protoplasts (single cells without walls) and forming single-cell suspensions after wall regrowth.

#### Future Plans

Studies are already underway to reduce cluster size by the action of hormones or enzymes, and will be presented later in this report in the section designated as New Studies. Our goal is 100% efficiency in the use of callus material for inocula, as well as the employment of the most effective cluster size for maximum embryogenesis.

#### EMBRYOIDS FROM DOUGLAS-FIR SUSPENSIONS

##### Introduction

The major objective of both feedback and empirical research has been the production of embryooids and their development into true embryos that will grow into plants. We have photographic documentation of de novo embryooid initiation after 3-4 weeks in Douglas-fir suspension cultures which had no differentiated cells at the start of the experiment. However, some of our cultures normally produce a low frequency of embryooids in the callus, and these may only enlarge somewhat when placed in the suspension cultures. Even in these, though, the final frequency of embryooids is usually greater than the starting frequency, indicating de novo embryogenesis in suspensions.

The main problem during the several years we have worked with conifer suspensions has been that we can initiate embryooids up to fairly high frequencies that consist of relatively few cells (3-20), but then they generally turn brown and die before enlarging further into true embryos. The results of electron microscopy studies have shown that the brown buildup is probably phenolic in



nature, and probably inhibitory to further growth and differentiation. We have found that greening can be stimulated by the addition of polyamines and other chemicals, but the phenolics still apparently have been preventing complete embryogenesis. We have, therefore, formulated a hypothesis stating that phenolic buildup inhibits further embryogenesis. This is discussed in more detail elsewhere in this report under Working Hypotheses. As a result of this hypothesis, our new approach is to divide suspension embryogenesis into two phases.

Figure 28 illustrates the two phases of embryogenesis, of initiation followed by development. Using this new approach, we now have started new studies to test different factors for initiating embryoids that will be used for further development of embryoids into embryos. Our assumptions are that factors that stimulate embryoid initiation probably also stimulate phenolic buildup and, hence, inhibit development. The purpose of the developer medium will be to permit embryoid enlargement without phenolic buildup. If phenolics do inhibit further embryogenesis, then without phenolics we should see complete embryogenesis.

The purpose of this section is to focus on embryogenesis in suspension cultures, and examine some of the problems and new approaches to solving the problems. Rather than duplicate a large amount of information, references will be made to a paper given at a recent TAPPI meeting and reproduced in the appendix.

#### Methods, Materials and Results

The methods have been detailed elsewhere in this report, in the several earlier sections dealing with suspension cultures. Embryoids have been initiated de novo in suspensions of small cell clusters, and probably initiated in larger fractions. Embryoids have also been enlarged up to 100-200 cells from embryoids that were possibly preformed in the callus and only developed in liquid suspensions.

Many different treatments have produced low frequencies of embryoids, but only a few treatments have produced embryoids comprised of up to 50-60% of the cells. Although embryoids of other species have been observed in suspensions, only Douglas-fir embryoids will be considered in this section.

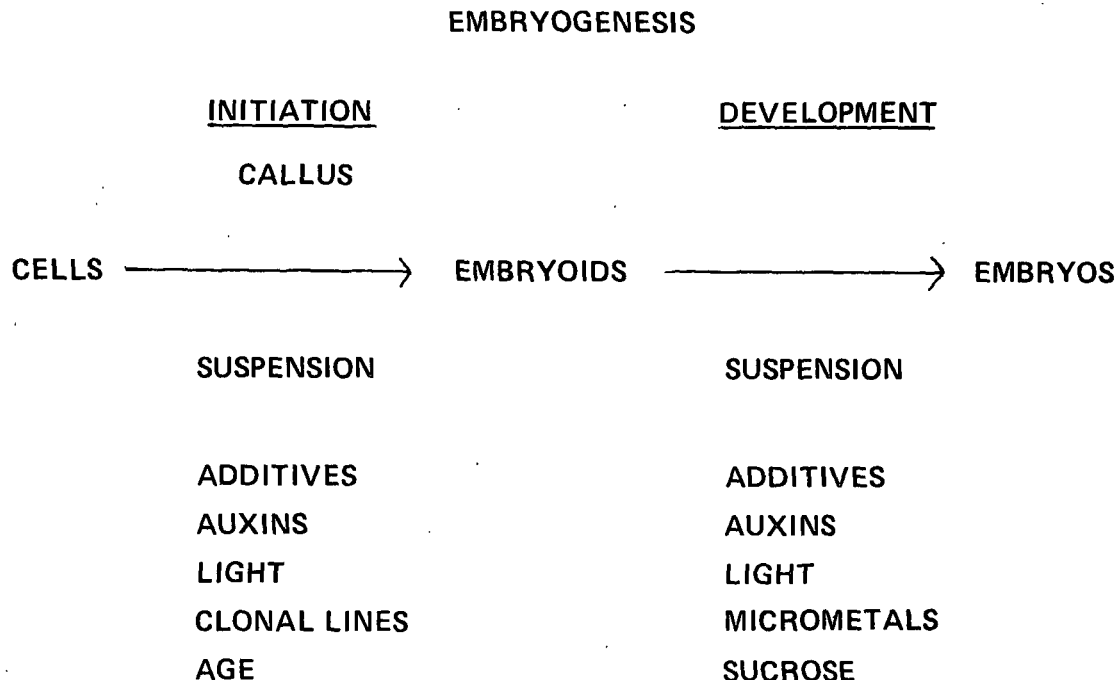


Figure 28. Illustrated Are the Two Phases of Embryogenesis, of Initiation Followed by Development

#### Discussion

On the basis of intensive examination of conifer suspension cultures over the past 6-7 years with a Zeiss light microscope, as well as the improving health of the structures in the past few years, Winton is convinced that what he calls embryoids show definite embryonic tendencies. None have ever reached the globular, heart or torpedo stages, and relatively few of the largest observed have had a bipolar appearance. However, the vigor and direction of development indicate a nonrandom sequence of cell division that appears to be trying to develop into an embryo.

According to Winton's view (see TAPPI paper in the appendix), embryonic structures arise from single cells, either from a small cluster of cells or free floating without an associated cell cluster. In contrast to this view, most workers studying embryogenesis in herbaceous species show the embryo developing from the cell cluster at the end of a filament of linear cells. According to their model, Winton is looking for embryogenesis at the wrong end of the filament.

It may very well be that conifer embryogenesis in suspension cultures will follow the same developmental pattern as in carrot and other herbaceous species, that of developing from the cell cluster. However, embryogenesis has not yet been completely documented in any conifer species, so it is premature to comment now on the origin of embryos. One or both points of origin may prove to be correct. The main argument supporting Winton's view seems to be the observed development of the so-called filament end in his conifer suspensions, with no development yet of the cell cluster at the other end. Also, Winton's embryoids occur either with or without an associated cell cluster. Some of the best structures presented by Winton had no attached cell cluster. On the other hand, he has found filamentous Douglas-fir structures with cell clusters similar to those found by Durzan and Steward (16), in suspension cultures of white spruce and jack pine. However, none have developed further in either lab.

Both routes are being considered in new studies being started, and whichever route proves to be correct will be utilized for the mass production of conifers from suspension cultures. Winton has stressed the filament-embryoid model because these structures have been most prominent in his suspensions. However, both models should be held as possibilities, and efforts made to try to design experiments that will recognize structures originating from either end of the filament.

### Future Plans

New studies have been started to provide separate media for initiation and development of embryoids. This approach will test the hypothesis that if phenolics can be reduced or eliminated then embryos will develop from embryoids. We will also attempt to isolate both types of embryoids as developmental media are improved, in order to study the growth of individual structures into embryos. Preliminary isolation has demonstrated the workability of some known techniques, and some new methods have been tried. Now we need embryoids that have developed sufficiently to be embryogenic, yet without phenolic buildup. Since the production of embryoids is the major objective of this program, this study will continue.

### ULTRASTRUCTURE OF DOUGLAS-FIR EMBRYOIDS

#### Introduction

The efforts of the Electron Microscopy Laboratory in the past several months have centered on the problem of deciding whether or not cell clusters referred to as "embryoids" in the Tissue Culture Laboratory are, indeed, embryo-like or at least appear to possess the capability for embryolike activity.

The above information is an important aspect of our overall project data bank at this time, since it will aid in evaluation of the degree of success as well as perhaps the validity of striving to achieve and maintain "embryoid" producing techniques. The latter are devised and modified by collaborative efforts of the Tissue Culture and Biochemistry Laboratories.

Primary objectives in our cytological investigations were to assess the general state of health of the cells concerned, note their physical relationship to one another, and to offer a prognosis as to whether or not the "embryoids"

appear to be capable of further development along the same lines as conventional, true embryos.

Feedback information from the above observations is expected to be used in the other laboratories to gauge to some extent their success in experiments designed to produce large numbers of viable embryoids.

#### Methods and Materials

Cell clusters in Douglas-fir callus of the type routinely labeled "embryoid" by the Tissue Culture Lab were isolated by Lawson Winton and put into fixative provided by the EM Lab. Subsequently, processing and microscopy of these cell clusters were then carried out in the latter facilities.

Careful embedment and sectioning of individual cell clusters permitted adjacent ultrathin and semithin sections of these specimens to be cut for correlative EM and light microscopy, respectively. Details visible and noted in the light microscope were then easily studied at much higher magnification in the transmission electron microscope (TEM).

#### Results and Discussion

As a general observation, all the "embryoids" we have examined thus far do not appear to have the potential for further development. Most of the cells in the E-cell clusters are quite highly vacuolated, and contain a substantial amount of tannin phenolics. Also of considerable importance was the observation that cells making up the "embryoids" are encased by well-developed mother cell walls. There were a few apparently healthy cells also encountered in some of the embryoids, but these were usually surrounded by the other, obviously non-meristematic cells.

As a check on our fixation procedure, we processed in a similar fashion, specimens of some commercially obtained spinach leaves. Ultrastructural preservation in the latter (Fig. 29) was as good if not better than most seen in the embryoids, indicating a high degree of confidence in our preparation techniques. The assumption that the true state of the embryoids was being reflected by our techniques was further supported by the detection of a few healthy and well preserved cells in clusters of several that were apparently moribund.

Figures 30-37 illustrate the typical cytoplasmic state of the embryoids as viewed in the light and electron microscope.

In Fig. 30 is shown an isolated, embedded embryoid before sectioning. The same embryoid is illustrated in Fig. 31 after sectioning, revealing what appears to be an organized cluster of cells to actually be a doublet of two very large and extremely vacuolated cells. A similarly sized embryoid is depicted in Fig. 32, which does consist of several cells, at least seven, and all within a relatively intact mother cell wall. This cluster might have continued development had it not accumulated tannin deposits sufficient to almost fill every cell.

The cytoplasm of the cells in the latter embryoid was in an overall state of degeneration, not as bad as that of the embryoid in Fig. 33, but definitely in a condition implying destruction.

Perhaps the embryoid that appeared to have the best potential of all the ones we examined was the one in Fig. 34. This cluster was composed of probably 13+ cells, all containing minimal amounts of tannin. However, a relatively thick cell wall encased many of the cells (arrow), and all cells were becoming highly vacuolate. The cytoplasm of these cells was, for the most part (Fig. 35), not really in a healthy state, and in all likelihood, this embryoid was harvested

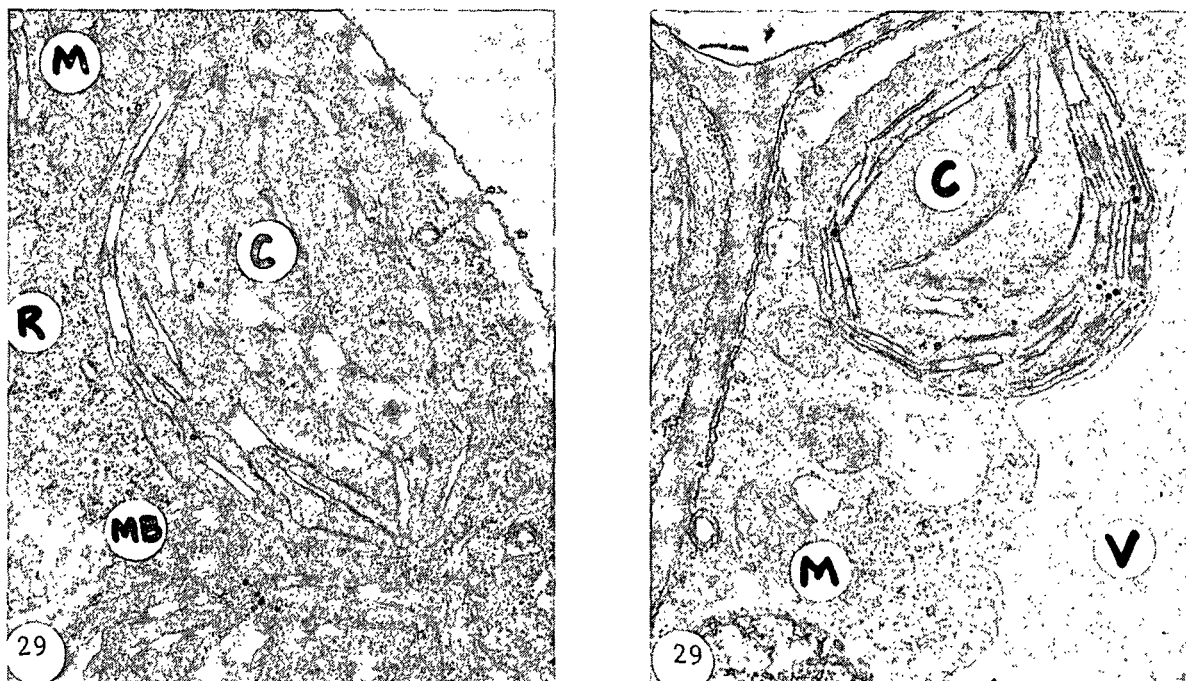


Figure 29. Portions of Two Cells from Spinach Leaves Fixed by the Same Procedure as the Embryoids. Note Good Preservation of Chloroplasts (C), Mitochondria (M), Microbodies (MB), and Ribosomes (R), Even Though the Cells Are Highly Vacuolated (V). 16,000X

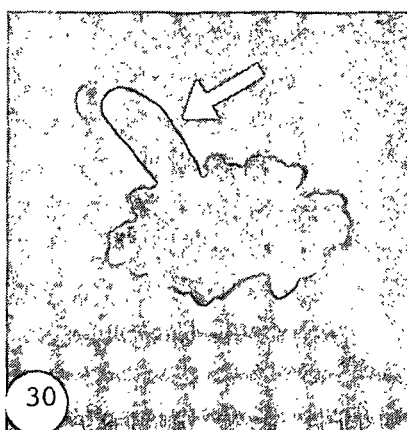


Figure 30. Embedded Embryoid Prior to Sectioning for Light and Electron Microscopy. Note the Elongated Portion of the Specimen Suggestive of an Embryoid-type Region (Arrow). 75X

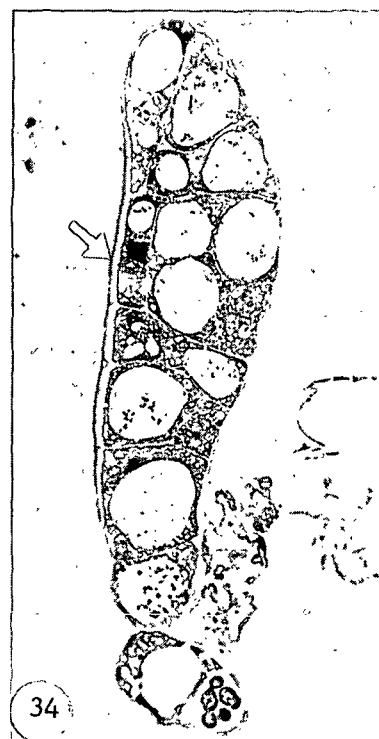
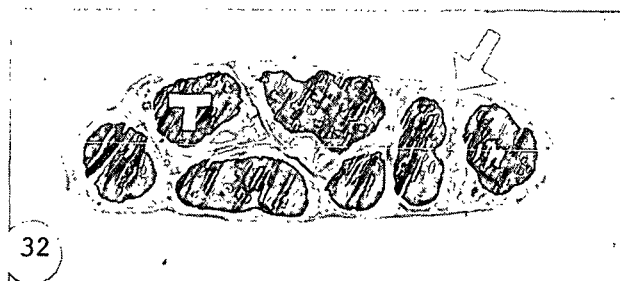
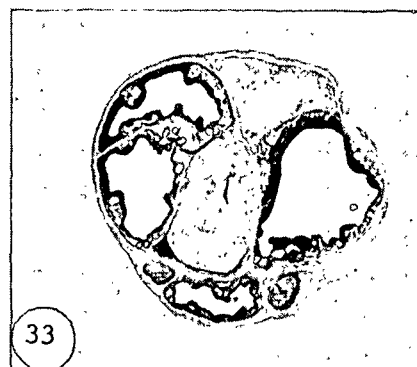


Figure 31. Semithin, Longitudinal Section of the Embryoid in Fig. 30 Showing the Latter to Consist of Only Two Large and Highly Vacuolated Cells. 300X

Figure 32. Embryoid Composed of Highly Vacuolated and Tannin-filled Cells (T). Note the Mother-cell Wall Encasing These Cells (Arrow). 300X

Figure 33. Cross Section of an Elongated Embryoid Showing Several Badly Degenerated Cells with Tannin-lined Vacuoles. One Apparently Healthy Cell Remains in the Center of the Embryoid. 300X

Figure 34. Elongated, Bipolar Embryoid with Several Only Moderately Vacuolated Cells. TEM of Those Cells, However, Showed Them to be Degenerating (See Fig. 35). Note the Well-developed Cell Wall (Arrow). 300X



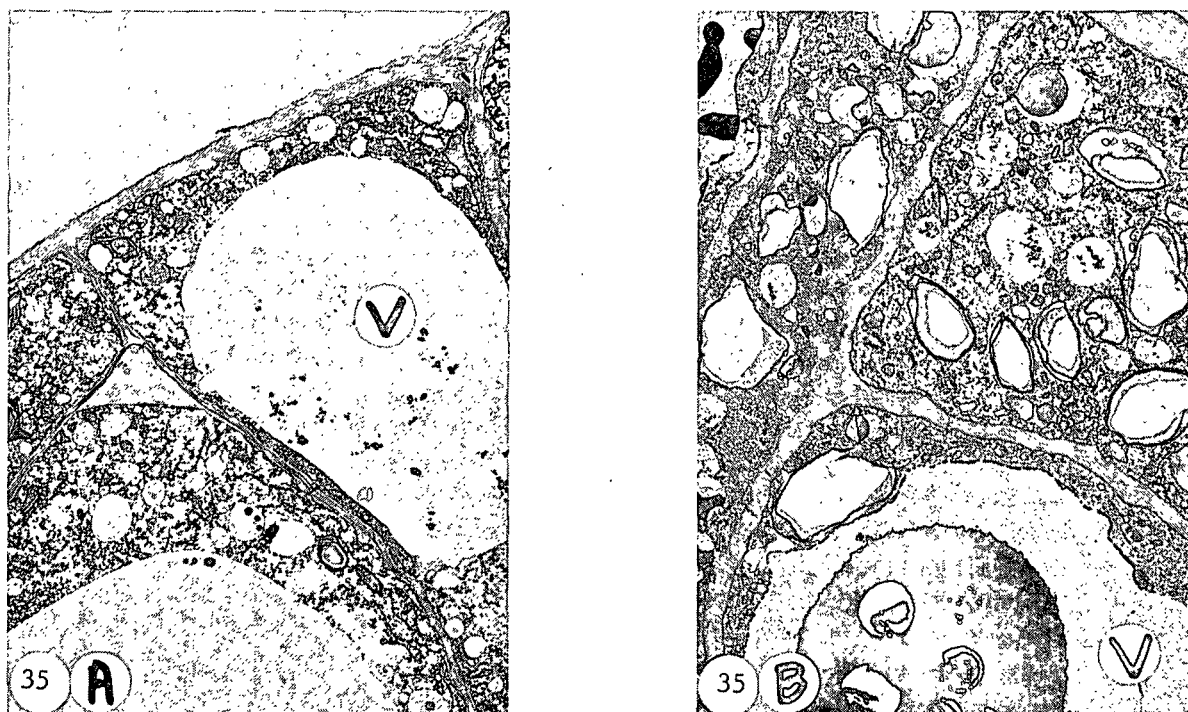


Figure 35. Electron Micrographs of Typical Cells from Fig. 34 Showing a Badly Degenerating Cell (A) and Some that Are Only Starting to Degenerate (B). While All the Cells Were not Extremely Vacuolated (V), the Cytoplasm Itself Showed Definite Signs of Initial Destruction. 1700X

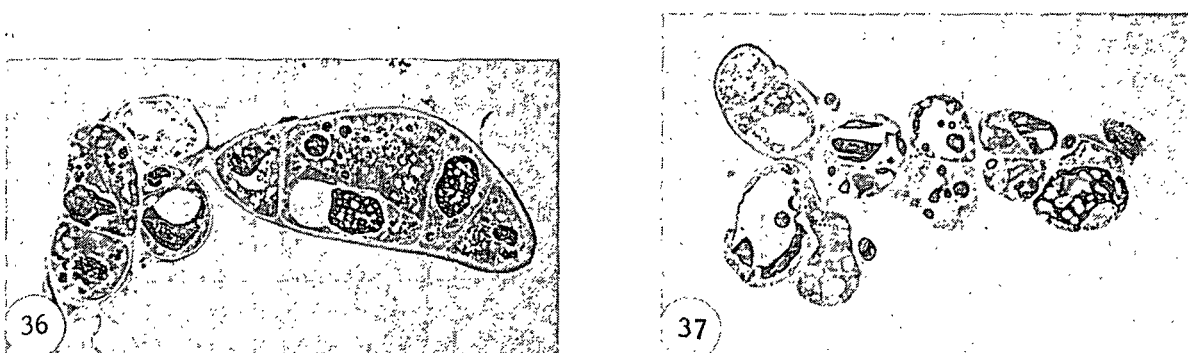


Figure 36-37. Two Embryoidlike Structures Showing Some Slight Signs of Bipolarity but Containing Mostly Moribund Cells, as Confirmed from Electron Micrographs of the Same Specimens. 300X



Figure 38. Embryoid Produced by UDP and Pyrophosphate  
in Sequential Feedback. 280X

just prior to undergoing changes that would render it very similar to that in Fig. 32.

Two more embryooids are shown in Fig. 36-37. In Fig. 36, a few of these cells seemed reasonably healthy (see also Fig. 37). However, again, the cells are accumulating phenolics, are becoming highly vacuolated, and one embryooid (Fig. 37) is not even well organized. Two cells here (arrow) have extremely well-developed walls.

### Conclusions

From our investigations on the "embryooids" isolated by the Tissue Culture Laboratory, one finding of considerable importance was that the cells making up the embryooids are encased in a well-developed mother cell wall and are not just a cluster of cells held together by a matrix of intercellular

materials. Also, it appears that these cell clusters have reached a relatively stable condition, probably including cluster size and cell number. The main reasons for this halt in potential growth would appear to be related to the buildup of tannin (phenolics) in the cell vacuole, concomitant with increased vacuolation. Whether this accumulation of tannin is the precise cause of the cell degeneration or whether it only reflects other degenerative or catabolic processes within these embryoids is difficult to forecast from static microscopical observations of dynamic phenomena.

### Plans

Continued cytological observations hinge on the production of larger and better organized embryoids. Observations will be made as required and are expected to include assessment of the general physiological condition of the cells and the documentation of the development of meristematic areas and/or cells with specialized functions and the physical relationships between cells making up embryoids.

### FEEDBACK RESEARCH

#### Introduction

Feedback research is the testing ground where attempts are made to take insights from biochemical investigations and utilize them to induce or control growth and embryogenesis. Also covered here are what have come to be known as perturbation experiments which are based mainly on insights gleaned from the literature rather than from our own experimental observations. Such experiments are designed primarily to generate leads although, of course, induction of embryogenesis would be a very acceptable response should it occur.

Appendix Table VI illustrates the relationship between the feedback research and the other studies underway.

The feedback investigations reported below with UDP (uridine diphosphate) and associated additives were attempts to begin some logical additive combinations relative to the carbon utilization hypothesis (described under section on Working Hypotheses). Iron concentration reduction was instituted in relation to the peroxide regulation hypothesis and the fact that catalase and peroxidases are iron-proteins. The minor metals subtraction was perturbation research conducted in anticipation of results from elemental analysis of seedling and callus tissue. These results may be considered now as part of the feedback input for medium design. Malate and succinate were used not only as aerobic substrates but because of reported stimulatory and inhibitory effects on other metabolism (17). The perturbation agent, silver nitrate, was reported recently to be a potent antiethylene agent (18). As such, it relates indirectly to the peroxide regulation hypothesis and may be useful in that regard.

Other feedback agents related to the IAA oxidase and peroxide regulation hypotheses have found increasing use. The monophenol, p-coumaric acid, was used as a promoter of IAA oxidase while catechol, a diphenol, was used as an inhibitor. Scopoletin has been used in both roles with its function dependent upon its concentration (19). Quercetin and taxifolin, a close relative found naturally in Douglas-fir, have been used in feedback trials; quercetin (or derivatives) is reported to be an IAA oxidase inhibitor but also is known to block ATPase and phenylalanine ammonia lyase (PAL) (20-22). Glycolic acid has been added as a possible hydrogen peroxide generator in vivo, while commercial catalase has been added in some tests to destroy or utilize peroxide. These latter two additives, along with several other chemicals, have been tested against cell suspensions and are tabulated

below as perturbation agents even though in many cases probable links to the main hypotheses could be established to justify calling them feedback agents.

It is well to reiterate here that the use of these chemicals in vivo may elicit responses unrelated or only partly due to effects on reactions that the investigator had in mind. For example, it was mentioned above that quercetin is known to inhibit at least three different enzymes in vitro, but what it will do at a given concentration in vivo is no doubt subject to several environmental restraints. Also, there is no guarantee (although checks can be made up to a point) that added chemicals remain unchanged when added to cells and act in the original form in which they are added. A good example (not proven) of this might be catechol which, since phenol oxidase activity is known to be present, is probably converted rapidly to toxic quinone in vivo; therefore, one must be very careful and not assume without reservation that it remained catechol and acted as an IAA oxidase inhibitor.

#### Methods

These experiments were run as usual in 10 ml suspension cultures on roller drums as detailed elsewhere in this report. The medium was 26-10, 26-10 without Mn, or 19-U (urea-based). Concentration ranges for some additives discussed below were: UDP, 0.1 mM; Fe, 0.7-5.6 mg/liter; malonic acid, 1 mM; succinic acid, 0.5-5 mM; malic acid, 0.5-5 mM; pyrophosphate, 0.1-10 mM; and fluoride, 0.1-10 mM. Concentrations of other chemicals are listed in context.

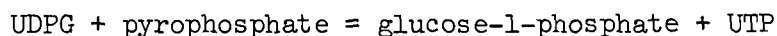
#### Results

##### Carbon Utilization Hypothesis — UDP and Related Additives\*

In some of the early progress reports it was noted that sucrose synthetase activity was apt to provide a major route for the entrance of sucrose carbon

\*Hypothesis described in detail in section on Working Hypotheses.

into callus or suspension cell metabolism. It was shown, for example, that all sucrose disappeared from the growth medium within a very short time if UDP\* was provided in the medium along with the sucrose. Also, the Ph.D. thesis research of Graham (23) demonstrated the presence of sucrose synthetase in the tree callus tissues. This reaction,  $\text{UDP} + \text{sucrose} = \text{UDP glucose} + \text{fructose}$ , brings glucose into the metabolism picture in an activated state so that no further energy investment is necessary for utilization. However, UDPG, once formed, may have more than one possible fate, e.g., it might serve as precursor for storage or structural polysaccharides which would delay the capacity to utilize the glucose for either biosynthetic or energy purposes. The most direct route for immediate utilization for dynamic functions of the cells would seem to be via the reaction catalyzed by UDPG pyrophosphorylase:



The sequence of sucrose synthetase followed by UDPG pyrophosphorylase would appear to be ideal from several points of view which will not be dealt with here.

Whereas UDP had been added to suspension cultures before, along with sucrose, lately we have been adding a further promoter of the "sucrose to UDPG to glucose-1-P sequence," namely, pyrophosphate (PPi). Low concentrations of fluoride (24) were sometimes added also in hopes of protecting the added PPi, but this is probably not specific enough to be very useful unless other interactions with the cellular system are also beneficial. Malonic acid (see below) has been used instead of fluoride recently to inhibit pyrophosphatase. The general trend has been that UDP is quite a consistent producer of sizable embryoids with the maximum effect appearing in about one week; thereafter, the old problem

\*See list of abbreviations in appendix.

of reversion sets in. The addition of PPI with the UDP also appears to be beneficial if one does not exceed about 0.1 mM. At times the combination of UDP plus glucosamine (see earlier reports) has out-performed either of these additives alone. An interesting sequence which has been run with some success is to treat for three days with UDP or UDP + glucosamine and then transfer to a medium with PPI + a pyrophosphatase inhibitor. The embryoid in Fig. 38 arose in this way. At this point we are in need of more information about the stability of the added pyrophosphate in vivo. Since the embryoids found from this type of feedback are often large but not numerous, it seems likely that one may be observing the development (enlargement) of preexisting small embryoids here rather than induction per se.

#### Iron Concentration

The iron level was varied initially because of its relationship with the iron-proteins, catalase and peroxidase. Several years ago, Dekock (25) reported differential effects on catalase and peroxidase tied to iron nutrition. Iron levels in our media had been at a fairly standard 5.6 mg/liter. This was found to be excessive, particularly in terms of greening of callus cells or suspension cells but also in terms of embryogenesis to a lesser extent. The optimum iron level appears to be about 1.4 mg/liter for both loblolly pine and Douglas-fir suspension cultures with perhaps slightly higher amounts for agar cultures. Reducing the iron levels not only promotes greening but also retards browning. A relationship between chlorophyll content and iron nutrition was also discussed by Dekock (25). Here, as in the case of manganese (below), there is likely to be a relationship between pH changes of the medium during culture and the concentration optimum, but this relationship has not been investigated.

#### Minor Metals Subtraction

Prior to elemental analyses of organized and unorganized Douglas-fir tissues, an experiment was started in which individual minor metals or combinations thereof were deleted from Douglas-fir stem callus suspension medium (26-10). This experiment also included some additions to 26-10, such as selenium. Cells were examined weekly under the light microscope for a period of 42 days. These results show some trends which will be useful in conjunction with the elemental analyses data, but they are mostly inconclusive as they stand alone. There may have been too much carryover from the callus inoculum to yield reliable data in this instance, i.e., effects may be more pronounced in the long term. In the future, when necessary, this problem will be addressed in conjunction with experiments considered in the elemental analysis section. Manganese has received additional study and many feedback and perturbation experiments now employ Medium 26-10 with lowered Mn concentrations as well as 1.4 mg/liter Fe.

#### Malate, Succinate, and Malonate

While these organic acids are well-known for their roles in aerobic metabolism, they can also exert regulatory effects at low concentrations. Lane (17) has reported stimulation of IAA oxidase by succinic and malic acids. Malonic acid, while famous as a competitive inhibitor of succinic dehydrogenase, has many far-ranging effects in some living systems (26). Included would be inhibition of pyrophosphatase. Neither succinate nor malate was very exciting as an additive although it may be notable that the not-so-high highest concentrations used (5 mM) had deleterious effects; at 0.5 mM malate promoted some embryoid development. To date malonic acid has been studied only at 1 mM with two iron levels. After 10 days samples with malonate were slightly ahead of controls in terms of



embryoid formation, but, in the absence of medium change, the situation underwent reversal over the subsequent week.

#### Silver Nitrate

Experiments conducted so far suggest that if silver nitrate is to be useful it will be at low concentrations (1 mg/liter or less) and as a pretreatment to other feedback additions. Used alone, it has shown no special promise.

#### Catechol, p-Coumaric Acid, and Scopoletin

These compounds are ostensibly inhibitors and promoters of IAA oxidase and were used in feedback experiments as though they would function in this manner. As already noted, the stability of these structures when added to the cells is not known and is, in fact, doubtful in the case of catechol. Catechol (500  $\mu\text{M}$ ) responded as a toxicant when used; since the response was quite rapid and the concentration substantial, the effect may have been direct through quinone formation rather than indirect via inhibition of IAA oxidase. Quinones in general were observed to be quite toxic.

Both p-coumaric acid and scopoletin were found to induce embryoid formation, usually in about one week but sometimes there was a second "wave" at about three weeks when cultures were retained that long and examined. Scopoletin was effective only at very low concentrations (0.5  $\mu\text{M}$ ) although instances were encountered where, at longer and longer times in culture, higher and higher concentrations of scopoletin were effective. This indicates that this compound may be gradually degraded in the system. According to the experience of Imbert and Wilson (19) with sweet potato cells, it appears that scopoletin was an effective inducer of embryoids under conditions where it would be expected to act as an IAA oxidase promoter. The validity of extrapolating results from sweet potato to

Douglas-fir is not yet established. Nevertheless, results with p-coumaric acid are consistent with that interpretation. This well-known IAA oxidase promoter was effective as an embryoid inducer over a range of 5 to 500  $\mu\text{M}$ ; strong solutions did become toxic. Of these three compounds, p-coumaric acid has been studied the most in feedback experiments. While it nearly always outperformed controls with respect to embryoid induction, the magnitude of the response was variable. Sco-poletin, used less frequently, has produced more and larger embryoids.

Embryogenic responses to these three compounds suggest that favoring conditions that promote IAA oxidation will lead to embryoid induction. The behavior of hydrogen peroxide and thiourea reported previously also points in that direction. At the very least, these results are compatible with the hypothesis that regulation of endogenous auxin concentration could be a significant factor in embryoid induction. For reasons already stated, the evidence must be considered circumstantial until supported or contradicted by additional research.

#### Quercetin and Taxifolin

Quercetin, a common flavonoid, is a reported inhibitor of ATPases, IAA oxidases (20-22), and PAL. With these attributes it might be expected to have both good and bad effects on embryogenesis from suspension cells. If indeed IAA oxidation is desirable to induce embryoid formation but not later on in development, quercetin might be a desirable addition to developer medium even though one might not want it present at the induction stage. In our hands it has had little effect on induction but did promote greening and general health of the cells for a week or so. Taxifolin, which is more water soluble, developed toxic effects sooner. Both compounds deserve further trials at lower concentrations (e.g., 50  $\mu\text{M}$  or less) in the developer medium. Also, their effects on specific enzyme

activities will be studied at several concentrations. On one occasion p-coumaric acid and quercetin were tried together and seemed to be a good combination but probably would be better used sequentially.

#### Perturbation Agents

Table V lists several compounds that have been tested recently as perturbation agents against Douglas-fir suspension cultures. Some of these compounds, like glycolic acid, are actually feedback agents that have not been extensively tested as yet. Except where noted in the table only one concentration was used so far, and in many cases this level was probably too high for suspension cell treatment. Where negative effects in particular were observed, further testing at lower concentrations should be considered. Some of the compounds, like glycolic acid, are quite promising as inducing agents while others may see service in developer media. Many of these and other chemicals tested over the past years are being used now in combinations and sequences.

#### Plans

The feedback and perturbation research will continue; however, much of it is expected to be related to the IAA oxidase hypothesis as long as it remains viable. Also, there will be a greater emphasis on development of embryoids in addition to initiation which has been the prime objective in the past.

TABLE V  
RESPONSE<sup>a</sup> OF DOUGLAS-FIR SUSPENSION CULTURES TO SOME PERTURBATION AGENTS<sup>b</sup>

Compound	Concentration	Observations <sup>c</sup>	Possible Functions	Comments
Abscisic acid	100 $\mu$ M	- 7 days	Growth inhibitor Development regulator	Further trials in developper medium
Methoxyhydroquinone	500 $\mu$ M	- 2 days (dead)	Toxic to enzymes	Oxidation product of diphenol
$\gamma$ -Guanidinobutyric acid	500 $\mu$ M	- then N.S.		From arginine catabolism
Methylornithine	500 $\mu$ M	N.S. then -	Inhibitor of ornithine decarboxylase	Blocks polyamine synthesis
Ribose	5 mM	+ 7 days	Nucleic acid metabolism	Not effective if P <sub>1</sub> also added
Catalase	1 mg/ml	- then N.S. (not consist- ent)	Destruction and/or utilization of peroxide	Erratic behavior suggests some peroxide involvement
MES buffer, pH 6.0	50 mM	- 7 days	Nonphysiological buffer	Relative of MOPS
Glutathione (reduced)	1 mM	- then N.S.	Reducing agent Cofactor of glyoxalase	Previously + in callus at lower concentration
Cysteine (free base)	1 mM	+ then -	Reducing agent Antioxidant for phenolics	
Mercaptoethanol	1 mM	N.S.	Reducing agent	
Glucosamine	1 mM	- then N.S.	Wall synthesis and intercellular adhesion	Promotes greening at lower concentrations
L-Proline	10 mM	- then N.S.	Wall synthesis	
Spermidine	1 mM	+ 7 days	Numerous	Polyamine promotes greening and longevity in callus
Indolaldehyde	1 mM	- 3 days		Possible IAA degradation product - needs more study
Polyvinylpyrrolidone	Saturated	N.S. ?	Binds phenolics	
Dihydroxyphenylalanine	1 mM	- 7 days (dead)		Diphenol
Sodium glyoxylate	1 mM	N.S.	Precursor of amino acids	Oxidation product of glycolic acid
Copper sulfate	0.1 mM	+ then -	Catalase inhibitor	
Ascorbic acid	0.2 mM	- 3 days (dead)	Reducing agent and oxidase inhibitor	
Glycolic acid	1 mM 0.1 mM	+ then - + then N.S.	Peroxide generator	Endogenously controlled peroxide source?
Maleate buffer, pH 6.0	0.1 mM	- & multi- vacuolate then dead	Physiological buffer	May block Krebs Cycle
Bovine serum albumin	1 mg/ml	N.S.	Enzyme protectant	

<sup>a</sup>Two weeks.

<sup>b</sup>Refer to abbreviations list.

<sup>c</sup>N.S. = no significant effect, + = improved appearance, - = deteriorating appearance.

#### STUDENT RESEARCH

With the expansion of the tissue culture program, interest in this area of research by biologically and biochemically oriented Institute students has greatly increased. Several students have recently completed and/or have under way research problems (A-291) or Ph.D. thesis programs that are expected to produce results that will expedite Project 3223 progress. Given below are summaries of student research that is either presently under way or has recently been completed.

Kim Robinson (Ph.D. thesis) - The title of Kim's thesis is the "Site and Nature of Microfibril Synthesis at the Surface of Douglas-fir Protoplasts." This work is expected to increase our understanding of the cell wall regeneration process for protoplasts produced and grown in a tissue culture system. The work is just getting under way with the development of experimental procedures for freeze-fracture and/or freeze-etching of protoplasts being the aspect of the program presently being considered. Dr. Russell Parham is chairman of Kim's thesis advisory committee.

Michael Smits (Ph.D. thesis) - Mike's thesis title is "The Role of Glyoxalase and Methylglyoxal in the Growth and Development of Cultured Gymnosperms." The investigation is based upon the hypothesis that cultured tree callus is a proliferating system that may be induced to organize (undergo organogenesis) by slowing down cell growth. Methylglyoxal is a known inhibitor of cell division in animal systems and can be inactivated by glyoxalase enzymes. Low concentrations of methylglyoxal have been shown to inhibit seed germination and in a special study research problem (A-291) Smits demonstrated inhibition of aspen stem callus growth. Mike's thesis research will be concerned with determining

the role glyoxalase enzymes play in the growth and development of conifer callus. Work on this program has been under way for about three months and presently preparation of radioactive methylglyoxal for use in anticipated enzyme studies is under way. Dr. Morris Johnson is Mike's thesis committee chairman.

Michael Mohler (A-291) - Mike completed his special studies research in March of 1977 and the title of his problem was "The Synthesis, Characterization and Auxin Activity of 3-Methylene-Oxindole (MEOI) and Related Compounds." MEOI is considered to be a major degradation product of the natural auxin indoleacetic acid (IAA). A major hypothesis guiding Project 3223 (IAA oxidase hypothesis) centers around the metabolism of endogenous IAA in cultured cells. Since 3-methylene-oxindole is not commercially available, Mohler's research, in which he succeeded in synthesizing both  $C^{14}$ -labeled and unlabeled forms, made it possible to study the role of this compound in IAA metabolism and the growth of cell suspensions. Preliminary tests failed to show MEOI had any effect on cell suspension growth at concentrations below 1 mM. Although the A-291 study is completed, further investigations with Mohler's and related compounds are anticipated. Dr. Morris Johnson was Mike Mohler's advisor on this study.

John Bobalek (A-291) - The title of John's special study, which was completed in March, was "Production, Isolation and Culture of Sterile Protoplasts of Douglas-fir." John was able to produce, isolate and culture sterile protoplasts but found only a few indications of cell division and possible callus formation. Basic methods were determined for filter sterilizing the enzyme solutions, washing enzymes from the protoplasts, and cleaning the protoplasts of debris. Robinson adapted Bobalek's method to his doctoral program and Dahl (see next) used information from both studies. John's work has been helpful as a starting point for other student research and will, without question, be helpful when project progress

reaches a point that protoplast fusion, as a method of producing conifer hybrids, becomes an important and active part of the Project 3223 program. Dr. Lawson Winton was John's advisor on this study.

Carl Dahl (A-291) - Carl's special studies research has the title of "Fusion of Douglas-fir Protoplasts." Successful fusion of protoplasts is the basis of somatic hybridization, which is one of the long-range goals of Project 3223. Knowledge of fusion techniques for Douglas-fir should be helpful when the approach is attempted with the southern pines. Carl is just completing his work in this area and was able to adapt routine methods used for crop plants and obtain a low frequency of fusion between Douglas-fir protoplasts. Fusion was also obtained between protoplasts of loblolly pine but protoplasts of Douglas-fir did not fuse with loblolly pine. Carl's work opened up several areas where additional student research could be carried out in the future. Dr. Lawson Winton was Carl's advisor on this study.

Mike Hanegraef (A-291) - Mike's special study has the title of "The Culture and Growth of Sterile Protoplasts of Loblolly Pine." Loblolly pine is the major species of southern pine and one of the conifers we are attempting to mass produce from suspension cultures. Loblolly pine will also be an important species for somatic hybridization using protoplast fusion. Success in this study would be a valuable step forward toward the production of pine hybrids. Mike has just finished his literature review and expects to start his lab work this fall. He plans to use the liquid media being developed by Dr. Waithaka for the continuous culture of loblolly pine cell suspension and modify the medium for protoplast culture, wall regrowth, callus growth and cell division. He may also attempt direct embryogenesis from protoplasts. Dr. Lawson Winton is Mike's advisor on this study.

## NEW STUDIES

The discussion that follows on newly initiated studies has been added in an attempt to keep the reader as up to date as possible on the total research program. Studies included in this section are ones in which work is under way, but not enough data have been obtained to warrant presenting the results in great detail or drawing anything but preliminary conclusions.

### PHENOLICS

#### Introduction

It has been recognized for many years (13) that monophenols stimulate IAA oxidase activity while further addition of hydroxyl groups to phenolic compounds results in structures that are IAA oxidase inhibitors. Examples of each have already been mentioned, i.e., p-coumaric acid as a promoter of IAA oxidase and catechol as an inhibitor of that enzyme. Scopoletin was found by Imbert and Wilson (19) to stimulate IAA oxidase at concentrations below 10 nmole/ml, but it became an inhibitor at higher concentrations. While the behavior of this compound against the IAA oxidase isozymes of the conifers has yet to be examined in detail, scopoletin was found to be an inducer of embryoid formation when applied at very low concentrations (see Feedback Research).

Cognizant of the role of phenols in the activity of IAA oxidase, we have reopened our investigations of endogenous phenolics in organized and unorganized tissues. Brief excursions have been made into this area before (see Progress Report Three, p. 30-33) as part of our general comparative analysis approach. Some of the difficulties inherent in such research were discussed at that time, and no attempt was made to identify any specific compounds. Because our extracts of conifer tissue to date suggest the presence of complex unstable mixtures,



identification of specific compounds remains an unreasonable goal in many cases. However, we do hope to pick out some very common structures and resolve classes of phenolics to some extent. Of particular interest are phenolic compounds that reportedly associate with IAA oxidase and influence the activities of IAA oxidase isozymes in vivo. Perhaps the major question could be stated as "Are the IAA oxidases in vivo in a stimulatory or an inhibitory environment relative to endogenous phenolics?" Of course, many of these compounds are presumably concentrated in the vacuole or are polymerized and not part of the living milieu. Nevertheless, smaller (physiological) amounts of these compounds no doubt exist in the cytoplasm and membrane systems which are reported to be sites of their biosyntheses (27).

The further study of phenolics at this juncture is, therefore, due to a possible strong bearing upon the IAA oxidase hypothesis. Specifically, our initial objective is to examine needles and needle callus for the presence of well-known promoters and inhibitors of IAA oxidase. Also undergoing scrutiny at the present time is the association of phenolics with our preparations of IAA oxidase from needles and needle callus.

#### Methods

Analysis of phenolics has proceeded along lines discussed previously (Progress Report Three, p. 30-33) and will continue to do so. However, different modes of extraction and chromatography are being used, particularly procedures described by Harborne (28) for various types of phenolics. Since many of these compounds probably exist as glycosides, both unhydrolyzed and hydrolyzed extracts are undergoing examination. Some commercial compounds are available for use as standards for chromatographic analysis. Well-resolved spots will be eluted and subjected to spectral analysis which may permit quantitation in some cases.

### Results to Date

Since work on phenolics resumed, extractions of Douglas-fir seedling sections and needle callus were conducted with ethanol and also with  $2N$  HCl. Chromatography in these cases was conducted on Whatman No. 1 and No. 3MM paper as well as on Gelman's ITLC sheets. These chromatograms reveal what appears to be an even more complex situation than previously envisioned. Extensive streaking was encountered on portions of the paper chromatograms. Cellulose TLC will be tried soon and may help to alleviate that problem. The most striking observation in this investigation so far is the presence of large amounts of putative cyanidin in hydrolyzed extracts of both needles and needle callus. The cyanidin concentration appeared to be considerably greater in the needle callus than in the needles in this particular comparison but no quantitative data are yet available. Several years ago Steward (29) reported that cyanidin was a cell growth inhibitor for carrot callus. However, the cyanidin in the conifer tissue obviously does not exist as such in vivo where another form may actually behave oppositely to the aglycone. Taxifolin seems to occur in both needles and needle callus extracts; many other compounds need better resolution. IAA oxidase preparations give reactions with reagents for phenolics even though these enzyme solutions have passed through gel filtration columns which remove unbound phenolics. The latter appear in the included fraction from the columns; such fractions typically undergo rather rapid oxidation (browning).

### Outlook

It seems very unlikely that all of these phenolic compounds are so compartmented within the tissues that they do not interact with enzymes such as IAA oxidase which, as indicated elsewhere in this report, has been found extracellularly as well as in cell extracts. The association of phenolics with the enzyme

preparations may be an artifact of preparation, but, if it can be shown that there is specificity of association along with influence upon activity of IAA isozymes, it could be suggestive of in vivo functioning.

In addition to the analytical work underway, the effects of selected commercial phenolics and of extracted phenolics (mixtures and resolved compounds) upon the conifer IAA oxidase activity will be compared. Similar testing for effects on growth and development will continue in Feedback and Perturbation Research (which see). Determination of the in vivo status of IAA oxidase relative to these compounds hopefully can be determined by use of degradation studies with IAA and GC analysis or with radioactive IAA.

#### IAA ANALYSIS

A confounding factor in much research concerned with the external manipulation of plant hormone ratios is the endogenous hormone level which often remains unknown. Very recently we have undertaken to determine the endogenous concentration of IAA and possibly related compounds in our callus and organized tissues. The IAA oxidase hypothesis requires some data on endogenous IAA if it is to have any validity. At this early date it can only be said that it appears that a gas chromatography procedure will provide the needed data. If so, it may be useful in following IAA metabolism perturbed by various agents in addition to determining steady-state levels of IAA in the organized and unorganized tissues.

#### POLYAMINES

Our previous perturbation and enzyme research suggested that polyamines may be affecting developmental processes in cultured conifer tissue. Furthermore, if polyamines are present along with the appropriate oxidases, they could be a

potential source of peroxide (see Peroxide Regulation Hypothesis). Although a very minor fraction of our total effort, polyamine analysis is beginning for the various tissues. If these results continue to suggest a role for these compounds, in embryoid initiation and development, metabolic studies will follow.

#### OPTIMIZATION OF LIQUID GROWTH MEDIUM FOR LOBLOLLY PINE SUSPENSION CULTURES

##### Introduction

The overall objective of this work is to develop an optimum liquid medium for cell suspension cultures of loblolly stem and needle callus. High yields of uniform green cells will justify the use of this material for investigations of metabolic regulation of growth and differentiation and detectable differences in growth and differentiation between cell cultures derived from different parts of the same plant. Since it is generally held that such cells are totipotent, it should be possible, in theory at least, to direct cells into a variety of developmental pathways by placing them in the appropriate environments. Such developmental capabilities have been shown to be species and even clone specific to various different growth environments.

Such medium is intended to provide continuous suspension cultures, in which the cells will rapidly achieve an equilibrium, become small and spherical, and divide periodically to produce a population of uniformly unorganized cells. Later, these cells will be subjected to different growing environments with the intent of inducing somatic embryoids which will eventually develop to plantlets.

The purpose of this section is to report early progress in this new study, and describe some of the approaches we plan to follow to promote the growth of loblolly pine cells in suspension cultures.

### Methods and Materials

Murashige and Skoog's (MS) liquid medium was used as a starting point and it was modified by adding L-glutamine, L-arginine, and L-asparagine at 100 mg/liter; NAA at 1.0 and 2.5 mg/liter, respectively, and with or without kinetin at 1.5 mg/liter to make medium MSK. The Institute-developed LV-10 medium with and without pyrophosphate (for greening) has also been tried, along with Medium F which is a modification of Gamborg's (30) B-5 medium. Media were periodically changed, usually every two weeks.

A standard rotating-drum/tissue culture tube growth system was employed. Drum speed was first set at 10 rpm and then later reduced to 1 rpm. The drums were placed in either low light (1000 lx) or in the dark, thus allowing both drum speed and light quantity to be evaluated.

Due to the rapid drop of pH in the media, several buffers have been tried. Cell examination was done with a Zeiss microscope at weekly intervals. Callus tissue was used that had previously been initiated and maintained, as described in an earlier section on loblolly callus cultures.

### Results and Discussion

Rapid growth of cell suspensions is defined here as an increase in cell numbers (volume). Different clones of stem callus tend to respond differently in their growth rate to a particular liquid medium. For instance, Clone 8 (LS-8), started on May 7 in LV-10 medium, has increased at least twenty times in cell volume. Cell cultures have been subcultured once due to the increase in volume. However, in other clones, e.g., 19 (LS-6) and 13 (LS-5), the cells in suspension turned brown and died within a week. Similar clonal differences in growth response to a particular medium have been reported by Cheng (4). In a recent experiment,

Clone 13 (LS-5) has shown rapid growth in F and MSK media, but we need a bit of time to clarify the reason. Needle cell suspensions haven't shown any improvement in any medium.

Conditions other than the growth medium have been observed to contribute to the increase in cell numbers. The speed of the drum which rotates the suspension cultures was the first to be noticed. After lowering the speed from 10-15 rpm to approximately 1-2 rpm, the cell volume increased rapidly within twelve days. Lower drum speed has been reported by others to influence cell aggregation and cell morphogenesis. On the other hand, free-floating single cells suspended in liquid medium at low densities have been reported to have very low capability of cell division. Such free-floating single cells have been reported to differ greatly in enzyme activities compared to those in aggregates.

The influence of light quality and quantity has not been studied in any great detail but low light intensity tends to favor improved growth of cell suspensions. More work is planned to study the influence of light quality and quantity on growth. So far the cells grown in the dark show no deleterious effects except for an observable chlorophyll degeneration. Cell division continues normally for those clones which respond positively to a particular medium.

Normally, within two days after starting a suspension culture, the pH of the medium drops from 5.8 to around 4.0. Such low pH may have an inhibitory effect on cell growth due to the pH influence on oxidation-reduction equilibrium and to the solubility of several heavy metals (Zn, Mn, Cu). When such metals become soluble at low pH, they have inhibitory effects on cell growth due to their interferences on enzyme systems in cell metabolism. Several buffers were tried (maleic anhydride, citrate, and morpholinoethane sulfonic acid), but they all had side effects on cell

growth, or did not maintain the pH in the desired range (5.0-6.0). At present, all cell suspensions are grown in unbuffered media, meaning that the pH problem is still unsolved. The concentrations of the heavy metals are not believed to be high enough to be toxic at these low pH's. In the future, we intend to run an elemental analysis of cell suspension cultures to investigate the effect of pH on elemental concentrations both in the medium and in the cells.

At the moment, little change has been made in media composition from the regular MS medium. Some additives, such as L-glutamine, L-asparagine, L-arginine and casein hydrolyzate have been included in MSK and F media, and a discussion of the effect of each individual additive on cell growth is planned for the next progress report.

#### Future Plans

We intend to continue subculturing clones which show good growth in any particular medium. Our main objective will be to develop a medium which will support good growth of all clones. This will mean evaluation of media which support cell growth of particular clones in a particular environment. We plan to evaluate the influence of additives on suspension growth, and whether the influence of any particular additive is common to all clones of both needle and stem explant origin. Studies on the effect of light quality and quantity on growth of suspension cultures will be continued. This will involve growing cultures under various light regimes and even some in the dark.

#### WASHING OF SUSPENSION INOCULA

#### Introduction

In some of the earliest feedback experiments, Johnson dropped water onto callus cultures and produced a few embryoids in the callus. Preliminary experiments

at that time also indicated that washing cells with water or media seemed to remove inhibitors of growth and differentiation. Now that we are using smaller cell cluster sizes for embryoid initiation, comparisons are being made in the preparation of inocula either with or without washing with water or simplified medium. Early results are reported here.

#### Methods and Materials

Various fractions of callus were used, ranging from 37-500  $\mu$ m, prepared from Douglas-fir needle callus with no differentiated cells, as well as some callus with embryoids preformed in the callus. Most cells were used for suspensions placed in the dark, but some were also placed in a light incubator. So far, single-distilled water has been used in comparisons with Medium 26-P (Medium 26-10 with added pyrophosphate and a sparing agent). We are just starting comparisons using double-distilled water (last distillation with permanganate).

#### Results and Discussion

Using single-distilled water for rinsing, cells lost more green color than when Medium 26-P was used. However, the number of new cells produced by cell division was about the same after a week. More dead cells seemed to plasmolyze in water than in medium, which may be beneficial in determining the frequency of viability. As yet we cannot say if distilled water or medium rinsing improves embryoid initiation better than not rinsing. More evaluation is needed. Also, we anticipate a better response to double-distilled water than to distilled water, but as yet have no results.

#### Future Plans

We plan to continue this study, testing medium against single- and double-distilled water for rinsing or washing callus cells for suspension inocula. We also plan long-range growth tests in water alone.



## CELL SEPARATION BY HORMONES OR ENZYMES

### Introduction

The most serious problem at this time in the suspension method is the high variability of results and the relative inefficiency of obtaining small cell clusters from callus cultures for inocula. The optimum development will be the production of single-cell suspensions, which is the goal of this study. We hope to be able to cause cells to dissociate from one another, either under the influence of hormones or enzymes. Hormones would cause the cell walls to grow in such a manner as to reduce wall adhesion between cells, thus forming smaller cell groups. Enzymes would also cause smaller clusters to form, mainly by the degradation of cell-cementing pectins by the use of pectinase. Another approach would be to use cellulase to remove cell walls to form protoplasts, then regrow the walls to form single cells in suspension.

This section discusses preliminary results of this approach.

### Methods and Materials

Some experiments have been run using low levels of pectinase with lower levels of cellulase. The purpose was to either reduce the number of cells per cluster, or to remove cell walls slowly and allow them to regrow to form single cells. Osmotic pressures were increased in media made with enzymes to reduce cell destruction when walls were removed. Best media were used that have been adapted by students in their protoplast research. These media will not be described here, since they are part of ongoing student research.

### Results and Discussion

In our preliminary experiments in the dark, using pectinase and cellulase, a combination of small cell clusters and single cells were produced within

24 hours. However, wall regrowth and cell survival was poor when either the original enzyme solution was left in the suspensions or the medium was replaced with fresh medium without enzymes. Much more work will be needed if we choose to spend more time on this approach. We have not yet started experiments using very high levels of regular auxin or an array of levels of exotic auxins.

### Future Plans

Because of the several problems seemingly associated with using enzymes, we may decide to let students work in this area. However, we will probably run a few more experiments to see if we can reduce the size of cell clusters with enzymes. This in itself would help solve the problem of inefficient callus use. If we continue to have problems getting cell walls to regrow, we will also abandon the use of protoplasts to get single-cell suspensions and let students work out this approach. Our main work will probably be with auxin levels to reduce cluster size, but we do not yet have all the auxins we wish to test.

## ISOLATION AND GROWTH OF EMBRYOIDS

### Introduction

After the spring project meeting with the RAC Subcommittee, the report of the Subcommittee suggested that we isolate and grow embryoids, to determine if the structures we call embryoids really have embryonic potential, i.e., will eventually grow into embryos and plants. At that time it was not clear what conifer embryoids should look like because, although embryogenesis had been described for herbaceous species, no one had worked out the developmental sequence for conifers. Durzan (16) found structures in suspensions of white spruce and jack pine that were similar to those found in carrot suspensions in Steward's and Halperin's laboratories, comprised of a thin cellular filament with a cluster

of small cells at one end. Durzan and others feel that the cell cluster is the embryonic end, from which the new embryo forms. Evidently, some feel that what we call embryoids are really the filaments, and that the embryonic cells are the cell cluster at the end of our filaments and not the filament itself. They may be correct. However, no one has ever produced a true embryo from suspensions of conifers, so we do not know which end will produce the embryo. One thing favoring Winton's model is the extensive cellular development of the "filament" end, up to fifty cells or more with cells attached at the base, and over 200 cells without attached cells. Unfortunately, Winton's embryoids turn brown and die after awhile, possibly from an accumulation of phenolics.

A number of different methods were tested for isolating cells and embryoids for continuous microscopic monitoring, but most embryoids eventually died in culture. When the electron microscopist reported that the embryoids were accumulating phenolics in lethal doses, we suspended isolation experiments until we could do more work with the new approach of initiator-developer media. Continued isolation was not justified until we could be sure the embryoids would remain free of phenolic buildup. As soon as we observe the initiation of embryoids, followed by transfer to a developer medium that reduces phenolic buildup, we will then return to isolating embryoids in that medium and monitor their growth in microculture.

The purpose of this section is to describe various microchambers we have used to culture isolated embryoids.

#### Methods and Materials

The microchamber of Jones, et al. (31) was used to grow isolated embryoids, either left in dry or wet covered dishes in the lab. Several media were used. Embryoids were also placed individually or in groups at the bottom of multiple

holes in sterile plastic boxes, used for the culture of microorganisms by other workers.

Nurse cultures were established in Petri dishes on agar medium, made by placing sterile microfilters 0.45  $\mu$ m on the surface after they had been dipped in liquid medium. They were placed in the center of the dish, after the center callus piece had been removed from a callus culture. Isolated embryoids were placed on the filter as well as on the agar surface. Filters were also placed on the surface of agar without callus. All dishes were sealed with Parafilm-TM for incubation. Embryoids were isolated from suspension cultures, using a mouth suction tube attached to a drawn-glass pipette via a microfilter. Work was done under a binocular scope at 30X. Some embryoids were fixed for electron microscope analyses.

#### Results and Discussion

All of the first isolates, which were left in dry covered dishes, died. Some survived for several weeks when placed in dishes where the slides were laid on bent-glass rods and water was added. New embryonic cells were produced by division, enlarging the embryoid in one microculture. However, the embryoid did not enlarge past what we call a medium-sized embryoid, with 15-20 cells.

None of the isolates survived in the plastic boxes, and contamination was high in Petri dishes. Some nurse cultures survived the longest, for a month or so, but there was no evidence of embryoid enlargement. These may be the best to try again when a good developer medium is found. However, the presence of callus in the culture may be defeating the goal of isolating embryoids away from the presence of phenolics. We did not try coconut milk in the medium.

### Future Plans

At present we are not isolating embryoids, but are concentrating on the initiation-developer combination to initiate embryoids, then develop them into embryos away from phenolic buildup. When we find a good developer medium that permits the enlargement of embryoids without phenolic buildup, we will then resume isolating embryoids in that medium to document the growth of individual embryoids from small to large structures. We also hope to show the whole process from de novo initiation to embryo production in suspension, using isolation chambers. Such documentation will also show the correct end of the filament whence embryo-genesis developed.

#### FUTURE PLANS

Most phases of the Project 3223 research effort are expected to increase greatly during the coming year. Dr. Donald Durzan joined the staff on July 1, 1977 and his addition, along with the recent addition of postdoctoral fellows Dr. Francis Hsu and Dr. Kimani Waithaka, brings the total number of professionals working on various aspects of the program to 7 and the number of technical personnel to 4. Less time is expected to be required in working out basic experimental procedures and greater emphasis can be directed toward definitive studies aimed at developing biochemically reliable procedures for obtaining embryoids, stimulating them to develop into true embryos, and eventually working out procedures for plantlet production.

Research has now been completed on the development of methods for preparing sterile media, screening cultures to obtain small cell clusters, preparing suspension inocula, evaluating feedback treatments and working out methods for processing and examining the ultrastructure of embryoids. Over the short term, new research is planned to examine the carbon utilization hypothesis, enzyme functions relating to the IAA oxidase and the peroxide regulation hypotheses, peroxide analyses, IAA analyses, optimization of growth media for loblolly pine suspensions, polyamine metabolism, phenolic build-up, enzyme treatments to reduce cell cluster size, feedback research, and techniques for the isolation and growth of embryoids. Details are contained in the section on New Studies and under plans in those parts of the report covering this past year's results.

Briefly, the plans during the coming year include research that will enable the tissue culture team to:

1. Select tissues that are designated by industry in order to permit positive genetic gains.
2. Determine the site and origin of embryoid initiation (callus or suspension cultures), isolate embryoids and stimulate their development into true embryos.
3. Obtain microscopic evidence of the organizational structure of developing embryoids, including physical connections between cells and the development of cells having special functions. Compare organizational structure with similar natural processes.
4. Obtain evidence concerning the importance of maintaining proper cellular peroxide levels (peroxide regulation hypothesis) in the initiation and enlargement of embryoids.
5. Develop a near optimum loblolly pine liquid growth medium, improve existing biochemical feedback assay methods and use this information in embryoid initiation studies.
6. Develop methods for preventing phenolic buildups which appear specific to conifer tissue and are believed to be inhibiting embryoid enlargement.
7. Examine in more detail tested morphogenetic systems, e.g., evaluate levels of IAA and other growth hormones in tissue from various treatments and origins as part of the research on the IAA oxidase hypothesis.
8. Investigate factors involved in the preliminary scale-up of cell numbers so as to provide the base for future controlled work in morphogenesis.

#### ACKNOWLEDGMENTS

The authors are indebted to individuals from the various paper companies who helped formulate the direction of this program. Acknowledged also are the efforts of Kimani Waithaka, post-doctoral fellow, working on a growth medium for loblolly pine suspensions, and Francis Hsu, post-doctoral fellow, working on hormone levels in callus cultures. Special thanks also go to Shirley Verhagen of the Tissue Culture Laboratory, John Carlson of the Biochemistry Laboratory and Hilikka Kaustinen of the Electron Microscopy Laboratory for their assistance in carrying out many aspects of the program. Also acknowledged is the assistance of Marianne Harder in preparing this report.



LITERATURE CITED

1. Raghavan, V. [ed.] Experimental embryogenesis in vascular plants. Section II: Adventive embryogenesis. p. 349-408. New York, Academic Press, 1976.
2. Monaco, L. C., Sondahl, M. R., Carvalho, A., Crocomo, O. J., and Sharp, W. R. Applications of tissue culture in the improvement of coffee. In Reinert, J., and Bajaj, Y. P. S. [ed.] Plant cell, tissue, and organ culture. p. 116-20. New York, Springer-Verlag, 1977.
3. Winton, L., and Verhagen, S. Shoots from Douglas-fir cultures. Can. J. Bot. 55:1246-50(1977).
4. Cheng, T. Adventitious bud formation in culture of Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco]. Plant Sci. Lett. 5:97-102(1975).
5. Winton, L. Callus and cell cultures of Douglas-fir. Forest Sci. 18:151-4(1972).
6. Gove, J. P., and Hoyle, M. C. The isozymic similarity of indoleacetic acid oxidase to peroxidase in birch and horseradish. Plant Physiol. 56:684-7(1975).
7. Alvarez, M. R., and King, D. O. Peroxidase localization, activity, and isozyme patterns in the developing seedling of Vanda (Orchidaceae). Am. J. Bot. 56:180-6(1969).
8. Srivastava, O. P., and van Huystee, R. B. Evidence of close association of peroxidase, polyphenol oxidase, and IAA oxidase isozymes of peanut suspension culture medium. Can. J. Bot. 51:2207-15(1973).
9. Lee, T. T. Changes in indoleacetic acid oxidase isoenzymes in tobacco tissue after treatment with 2,4-dichlorophenoxyacetic acid. Plant Physiol. 49:957-60(1972).
10. Gaspar, T., Khan, A. A., and Fries, D. Hormonal control of isoperoxidases in lentil embryonic axis. Plant Physiol. 51:146-9(1973).
11. Hoyle, M. C., and Routley, D. G. Promotion of the IAA oxidase-peroxidase complex enzyme in yellow birch. In Bialeski, Ferguson and Cresswell's Mechanism of regulation of plant growth. Bulletin 12:843-50. The Royal Society of New Zealand, Wellington, 1974.
12. Hendricks, S. B., and Taylorson, R. B. Breaking of seed dormancy by catalase inhibition. Proc. Nat. Acad. Sci. USA 72:306-9(1975).
13. Kenten, R. H. The oxidation of indolyl-3-acetic acid by waxpod bean root sap and peroxidase systems. Biochem. J. 59:110-21(1955).
14. Sagisaka, S. The occurrence of peroxide in a perennial plant, Populus gelrica. Plant Physiol. 57:308-9(1976).

15. Halperin, W. Alternative morphogenetic events in cell suspensions. *Am. J. Bot.* 53:443-53(1966).
16. Durzan, D., and Steward, F. Morphogenesis in cell cultures of gymnosperms: some growth patterns. IUFRO Workshop, Helsinki, May 28-June 5, 1970, Section 22.
17. Lane, H. C., and King, E. E. Stimulation of indoleacetic acid oxidase and inhibition of catalase in cotton extracts by plant acids. *Plant Physiol.* 43:1699-1702(1968).
18. Beyer, E. M., Jr. A potent inhibitor of ethylene action in plants. *Plant Physiol.* 58:269-71(1976).
19. Imbert, M. P., and Wilson, L. A. Stimulatory and inhibitory effects of scopoletin on IAA oxidase preparations from sweet potato. *Phytochem.* 9:1787-94(1970).
20. Stenlid, G. Effects of flavonoid compounds on oxidative phosphorylation and on the enzymic destruction of indoleacetic acid. *Physiol. Plantarum* 16:110-20(1963).
21. Graziani, Y. Bioflavonoid regulation of ATPase and hexokinase activity in Ehrlich ascites cell mitochondria. *Biochim. Biophys. Acta* 460:364-7 (1977).
22. Iredale, S. E., and Smith, H. Properties of phenylalanine ammonia lyase extracted from *Cucumis sativus* hypocotyls. *Phytochem.* 13:575-83(1974).
23. Graham, L. L. Sucrose synthetase from triploid quaking aspen callus tissue. Doctor's Dissertation. Appleton, Wisconsin, The Institute of Paper Chemistry, 1975. 130 p.
24. Naganna, B., Raman, A., Venugopal, B., and Sripathi, C. E. Potato pyrophosphatases. *Biochem. J.* 60:215-23(1955).
25. Dekock, P. C., Commisong, K., Farmer, V. C., and Inkson, R. H. E. Interrelationships of catalase, peroxidase, hematin, and chlorophyll. *Plant Physiol.* 35:599-604(1960).
26. Webb, J. L. Enzyme and metabolic inhibitors. Malonate. Vol. II, Chap. 1. New York, Academic Press, 1966. 1237 p.
27. Stafford, H. A. Relations between the development of adventitious roots and the biosynthesis of anthocyanine in first internodes of sorghum. *Plant Physiol.* 43:318-26(1968).
28. Harborne, J. B. Phytochemical methods. London, Chapman and Hall, 1973. 278 p.
29. Steward, F. C. Growth and organization in plants. Reading, MA, Addison-Wesley, 1968. 564 p.

30. Gamborg, O. L., Miller, R. A., and Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* 50:151-8(1968).
31. Jones, L., Hildebrandt, A., Riker, A., and Wu, J. Growth of somatic tobacco cells in microculture. *Am. J. Bot.* 47:468-75(1960).

#### RELATED PUBLICATIONS

Johnson, M. A., and Carlson, J. A. Attempts to induce embryogenesis in conifer suspension cultures: biochemical aspects. p. 25-9. TAPPI Forest Biology/Wood Chemistry Conference Papers, 1977.

Smeltzer, R. H., and Johnson, M. A. Evidence for the presence of cyclic adenosine 3',5'-monophosphate and its metabolism in loblolly pine (Pinus taeda L.) callus. *Can. J. Forest Res.* 7:68-75(1977).

Winton, L. Callus and cell cultures of Douglas-fir. *Forest Sci.* 18:151-4(1972).

Winton, L., and Huhtinen, O. Tissue culture of trees. In Modern methods of forest genetics. p. 243-64. Berlin, Springer-Verlag, 1976.

Winton, L., Parham, R., Johnson, M., and Einspahr, D. Tree improvement by callus, cell, and protoplast culture. *Tappi* 57:151-2(1974).

Winton, L., and Verhagen, S. Shoots from Douglas-fir. *Can. J. Bot.* 55:1246-50 (1977).

Winton, L., and Verhagen, S. Embryoids from suspension cultures of Douglas-fir and loblolly pine. p. 21-4. TAPPI Forest Biology/Wood Chemistry Conference Papers 1-5, 1977.

## GLOSSARY

Adventitious - Roots, shoots, embryos, or other organs or tissues developing in an abnormal position.

Amyloplast - A colorless plastic modified for starch storage.

Aseptic culture - Surface sterilization of parental explants, free from pathogens, but not necessarily free of internal symbionts.

Asexual reproduction - Reproduction without fertilization. New individuals may develop from vegetative parts such as tubers, bulbs, or rooted stems, or from sexual parts such as unfertilized eggs or other cells in the ovule.

Auxins - A class of plant growth hormones of diverse makeup which cause cell enlargement, apical dominance and root initiation.

Callus culture - Proliferation from a parental explant of many cells in protoplasmic continuity, but having no equivalence with any normal tissue. Same as tissue culture.

Cell differentiation - Internal chemical or ultrastructural changes preceding or accompanying specialization of function.

Cell suspension - Culture of single cells in moving liquid medium, often used incorrectly to describe suspension cultures of cells and cell aggregates.

Chloroplast - A membrane-enclosed subcellular organelle containing chlorophyll. Chloroplasts are the sites of photosynthesis. They contain DNA and poly-somes and can replicate.

Clonal propagation - Propagation of a group of plants derived from a single individual (ortet) by asexual reproduction. All members (ramets) of a clone have the same genotype and consequently tend to be uniform.

Coumarins - A class of phenylpropanoid phenolic compounds of which coumarin itself typifies the structures.

Cytokinin - A class of plant growth hormones associated with cell division, assisting with the transmission of the genetic information from the genes to the proteins.

2D TLC - Two-dimensional thin layer chromatography.

Developer medium - Used to grow embryoids into true embryos in suspension cultures.

Diploid - Having two sets of chromosomes in the nucleus. One-half of the chromosomes are contributed by one parent, one-half by the other parent. Many higher organisms are diploid except for their sex cells and associated tissue.

E-cells - Embryonic cells that have a relatively small central storage vacuole surrounded by a thick cytoplasm. Normal callus cells have a large vacuole and a small amount of cytoplasm.

EM - Electron microscope.

Embryo - The young plant developing in the megagametophyte from the fertilization of an egg cell, or without fertilization. In aseptic cultures, adventitious embryos show polarization followed by the growth of a shoot from one end and a root from the other end.

Embryogenesis - Initiation of embryoids or embryos from cultured cells.

Embryoid - A cell group approximating an embryo, but having a more random cell arrangement.

Empirical method - Method based solely on experiment and observation.

Enzyme - A protein molecule that catalyzes a specific chemical reaction.

ER - Endoplasmic reticulum. A system of membranes (originating from the external membrane of the nuclear envelope) that permeates the cytoplasm and which may or may not be covered with ribosomes.

Eucaryotic cells - Cells with true nuclei bounded by nuclear envelopes and which undergo meiosis.

Excise - Cut or isolate callus tissue from its parental explant, or to remove adventitious shoots from callus tissue for rooting.

Explant - A plant part excised and prepared for aseptic culture by surface sterilization followed by the exposure of live cells to a nutrient medium.

Fertilization - The normal union of two gametes during sexual reproduction.

Flavonoids - A class of phenolic compounds usually consisting of two hydroxylated aromatic rings joined by a three-carbon chain.

Gene - One of the units of inherited material carried on a chromosome; arranged in a linear fashion and indivisible.

Gene pool - Reservoir of genetic variability available for use in genetic improvement of tree species.

Genetic gains - Average improvement in progeny over the mean of the parents.

Genetic variability - The variation existing in a given population (species, for example) with respect to particular genes or arrangement of genes.

Genotype - The genetic makeup of an individual carried in the chromosomes.

Groundplasm - Homogeneous plasma (matrix) remaining after cell organelles and particles have been excluded.

Haploid - Having the reduced chromosome number, i.e., having one set of chromosomes in the nucleus. This is normal in sex cells, which have only half the number of sets occurring in diploid vegetative cells.

Hormone - Any growth substance which is generally transported to the site of action and can stimulate growth or cell enlargement (auxins), cell division (cytokinins), stem elongation (gibberellins), or can retard growth as in the abscission of leaves (ethylene).

Hybrid vigor - The increase in vigor, size and fertility of a hybrid as compared with its parents, resulting from the union of genetically different gametes and assumed to be due to special recombinations of dominant and recessive genes (heterosis).

Hybridization - The production of offspring of genetically different parents.

Hydrophobic - Water repelling.

Initiator medium - Used to initiate embryoids in callus or suspension cultures.

Inoculum - A small piece of tissue cut from callus, or a small amount of cell material from a suspension culture placed in contact with fresh medium for continued growth of the culture. Inocula (plural).

Interspecific hybrid - The progeny from matings between species.

Intraspecific hybrid - The progeny from matings within species.

Isozymes - Multiple forms of a single enzyme.

Lipids - Any of a group of biochemicals which are variably soluble in organic solvents and barely soluble in water.

Milieu - The whole chemical and physical environment of a culture.

Meristem - A localized group of cells, actively dividing and undifferentiated but ultimately giving rise to permanent tissue such as shoots, roots, wood or bark.

Meristemoid - A localized group of cells in callus tissue, characterized by an accumulation of starch, RNA and protein, and giving rise to adventitious shoots or roots.

Mitochondria - Small bodies in spaces of the ground cytoplasm. They are spherical, long rods, or threads, and are the sites of many important enzymatic processes. The inner layer of the wall is infolded into fingerlike processes.

Morphogenesis - Initiation of organized tissue in callus or suspension cultures.

Nonrelated species - Species that are members of different genera.

Nutrient medium - A solid or liquid combination of major and minor salts, an energy source (sucrose), vitamins, hormones, and occasionally other defined or undefined supplements. Usually made up from previously prepared stock solution, then sterilized by autoclaving or filtering through a micropore filter. Media (plural).

Organized tissue - Composed of regularly differentiated cells.

Organelle - A complex cytoplasmic structure of characteristic morphology and function, such as a mitochondrion or plastid.

Organogenesis - Initiation of roots or shoots from callus meristemoids.

Parasexual hybridization - Hybridization resulting from asexual fusion of cells, either diploid or haploid.

Passage - The duration of growth of callus or cell material from one subculture to another.

Plasmalemma - The semipermeable, unit membrane surrounding and containing the cell cytoplasm. In plant cells, it is pressed up against the inner surface of the cell wall.

Plantlet - A small rooted shoot. Also, a piece of callus having both roots and shoots that are not connected together inside the callus.

Polyploidy - Having three or more times the haploid number of chromosomes.

Procaryotic cells - Single-celled organisms and reproducing entities that lack a membrane-bound nucleus; they do not undergo meiosis; these include the viruses, bacteria, and blue-green algae.

Protoplast - Spherical cell protoplasm (cytoplasm + nucleus) bounded by a membrane but no cell wall.

Protoplast fusion - Union of two protoplasts into one cell.

Ribosomes - Macromolecules containing protein and RNA. They are seen as dense particles in electron micrographs. They are found in all types of cells in which protein is being synthesized.

SEM - Scanning electron microscope.

Somatic - Diploid body cells of an organism; those cells other than germ cells.

Subculture - Cutting solid callus into small cubes (inocula) for transfer to fresh medium. Sometimes used to denote the adding of fresh liquid medium to a suspension culture.

Suspension culture - Cells or cell aggregates dispersed and growing in moving liquid medium.

Tannins - A class of complex phenolic compounds known for their astringency and ability to tan the proteins of animal skins. There are two major types of tannins, the hydrolyzable and the condensed tannins.

TEM - Transmission electron microscope.

Tissue culture - General term for callus and cell cultures.

Ultrastructural - Sublight microscopic, intracellular structure.

Vacuole - A fluid-filled space in a cell. A single vacuole, taking up most of the volume of the cell, is present in many plant cells and contains a cell-sap which is isotonic with the protoplasm.

Vegetative cells - Nonreproductive cells such as haploid cells from female gametophytes of conifers or diploid somatic cells.

Vesicle - Small membrane-bound body in the cytoplasm.

Zygote - Fusion product of male and female sex cells or fusion product of protoplasts.

THE INSTITUTE OF PAPER CHEMISTRY

Lawson Winton

Lawson L. Winton  
Research Associate

Morris A. Johnson

Morris A. Johnson  
Research Associate

Russell A. Parham

Russell A. Parham  
Research Associate

Dean W. Einspahr mh

Dean W. Einspahr  
Senior Research Associate

Approved by:

John W. Swanson

John W. Swanson, Director  
Division of Natural  
Materials & Systems



RESEARCH PROGRAM FOR EMBRYOID INITIATION AND DEVELOPMENT, WITH TIMETABLE

	1976	1977	1978
	S O N D	J F M A M J	J F M A M J
I. TISSUE CULTURE <sup>a</sup>			
Environmental factors			
LP suspension growth			
Embryoid isolation			
DF embryoids & initiation-development			
II. BIOCHEMISTRY			
Carbon utilization hypothesis			
Peroxide regulation hypothesis			
Catalase-peroxidase studies			
Peroxide measurements			
Control of enzyme activity			
Enzyme interrelationships			
Elemental analyses			
IAA oxidase hypothesis			
Polyamine metabolism			
Perturbation experiments			
Hormone analyses			
Other hypotheses			
III. ELECTRON MICROSCOPY			
Enzyme localization			
Embryoid ultrastructure			
"On call" investigations			
IV. ALL LABORATORIES - FEEDBACK TESTING FOR EMBRYOID INITIATION AND DEVELOPMENT			
Carbon utilization hypothesis			
Peroxide regulation hypothesis			
IAA oxidase hypothesis			
Polyamine effects			
Elemental medium adjustment			
Other feedback			
V. REPORTING SCHEDULE			
Project (internal) and Progress (external)	Project Rpt.	Progress Rpt.	Progress Rpt.
Reports - on dates indicated	5, Jan. '77	5, July '77	6, July, '78

<sup>4</sup> Additional studies that were completed this past six months include: optimization of sterilization procedures, sucrose levels, cell cluster size, LP callus medium, and inocula preparation methods.

TABLE VII

COMPONENTS OF NUTRIENT MEDIA IN MILLIGRAMS PER LITER

Component	3 <sup>a</sup> & 10 <sup>a</sup>	19 & E-1 <sup>b</sup>	26-10	LV-10 <sup>c</sup>
NH <sub>4</sub> NO <sub>3</sub>	1,650	1,650	400	1,650
KNO <sub>3</sub>	1,900	1,900	4,000	1,900
MgSO <sub>4</sub> • 7H <sub>2</sub> O	370	370	185	370
KH <sub>2</sub> PO <sub>4</sub>	170	170	85	340
CaCl <sub>2</sub> • 2H <sub>2</sub> O	440	440	220	220
MnSO <sub>4</sub> • H <sub>2</sub> O	16.9	16.9	8.5	16.9
ZnSO <sub>4</sub> • 7H <sub>2</sub> O	10.6	10.6	5.3	10.6
H <sub>3</sub> BO <sub>3</sub>	6.2	6.2	3.1	6.2
KI	0.83	0.83	0.41	0.83
Na <sub>2</sub> MoO <sub>4</sub> • 2H <sub>2</sub> O	0.25	0.25	0.125	0.25
CuSO <sub>4</sub> • 5H <sub>2</sub> O	0.025	0.025	0.0125	0.025
CoCl <sub>2</sub> • 6H <sub>2</sub> O	0.025	0.025	0.0125	0.0125
Fe <sup>d</sup>	5.6	5.6	5.6	5.6
Nicotinic acid	0.5	--	--	0.5
Thiamine	0.1	--	--	0.1
Pyridoxine	0.1	--	--	0.1
Inositol	100	--	--	100
Asparagine	100	--	--	100
Casein	--	--	--	--
Adenosine	--	--	--	--
Sucrose	30,000	30,000	35,000	30,000
Agar	8,000	8,000	--	8,000
BAP	0.1	--	--	0.1

<sup>a</sup>Medium 3 contains 1 mg/liter NOAA and Medium 10 contains 5 mg/liter NOAA.  
NOAA — naphthoxyacetic acid; BAP — benzylaminopurine.

<sup>b</sup>Medium E-1 is Medium 19 plus 0.4 thiamine, 100 inositol, and 40,000 sucrose.

<sup>c</sup>Medium LV-10 contains 10 mg/liter NOAA and 100 tryptophane.

<sup>d</sup>5 ml/liter of stock solution containing 1.114 g FeSO<sub>4</sub> • 7H<sub>2</sub>O and 1.49 g Na<sub>2</sub>EDTA per 200 ml.

TABLE VIII

LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
arg	Arginine
BAP	Benzylaminopurine
cAMP	3',5'-Cyclic adenosine monophosphate
DAB	Diaminobenzidine
DF	Douglas-fir
E-cell	Embryonic cell
G-1-P	Glucose-1-phosphate
GC	Gas chromatography
IAA	Indoleacetic acid
IBA	Indolebutyric acid
IPA	Isopentenylaminopurine
KCNS	Potassium thiocyanate
LP	Loblolly pine
lx	Lux
MEOI	Methyleneoxindole
MES	Morpholinoethane sulfonic acid
MOI	Methyloxindole
MOPS	Morpholinopropane sulfonic acid
MS	Murashige and Skoog medium
MSK	Modification of Murashige and Skoog medium
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide (oxidized)
NADP <sup>+</sup>	Nicotinamide adenine dinucleotide phosphate (oxidized)
NBT	Nitrobluetetrazolium
NOAA	Naphthoxyacetic acid
PAL	Phenyl ammonia lyase
phe	Phenylalanine
PPi	Pyrophosphate
pro	Proline
TLC	Thin-layer chromatography
trp	Tryptophan
TrpAm	Tryptamine
tyr	Tyrosine
UDP	Uridine diphosphate
UDPG	Uridine diphosphate glucose
UTP	Uridine triphosphate

Paper presented at TAPPI Forest Biology Workshop, June 20.

# EMBRYOIDS IN SUSPENSION CULTURES OF DOUGLAS-FIR AND LOBLOLLY PINE

Lawson Winton and Shirley Verhagen  
The Institute of Paper Chemistry, Appleton, Wisconsin 54911

## ABSTRACT

Using a team approach, we are attempting to determine the trigger mechanism of embryogenesis in suspension cultures of Douglas-fir and loblolly pine. In order to evaluate biochemical feedback, we in the tissue culture laboratory have developed methods for (1) the preparation of inocula from young needle callus, and (2) microscopic, semiquantitative monitoring of cell growth and embryoid initiation. These methods will be discussed, along with some of the advantages and disadvantages of using this type of assay.

At least four types of embryogenesis have been reported among plants, based on differences in the site of embryoid initiation, development of embryos and growth into plantlets. For convenience, we propose the classification shown in Table I. In Type I embryogenesis, all stages remain associated with the explant and has been reported on stem segments of *Ranunculus sceleratus*<sup>1</sup>, epidermis of carrot<sup>2</sup>, hypocotyl segments of tobacco<sup>3</sup> and from pollen grains of tobacco<sup>4</sup>. Among tree species, embryoids were grown on stem segments of *Biota (Thuja) orientalis*<sup>5</sup> and *Abies balsamea*<sup>6</sup>. In Type II embryogenesis, all stages occur in callus cultures and has been reported in carrot<sup>7,8</sup>. In Type III, embryoids are initiated in callus and transferred to liquid where they develop further. However, there have been two major responses in liquid cultures. In Type III-A, disorganized callus masses develop in liquid and some produce roots, but shoots are not produced until the callus masses are transferred to agar medium, as has been reported in carrot<sup>9,10</sup>. In Type III-B, true bipolar embryos develop in liquid cultures and have been reported in carrot, first in conditioned medium<sup>11</sup> then in defined medium<sup>12-15</sup>. Embryo development has also occurred in liquid suspension cultures of endive<sup>16</sup>, asparagus<sup>17</sup>, lily<sup>18</sup>, and bromegrass<sup>19</sup>. Among tree species, embryoids have been observed in suspension cultures started from callus of *Picea glauca*<sup>20-22</sup>, *Pinus banksiana*<sup>20</sup>, *Picea abies*<sup>23</sup>, *Pseudotsuga menziesii*<sup>24,25</sup>, and *Pinus taeda*<sup>25</sup>.

In experiments with Douglas-fir<sup>24,25</sup>, we observed 60-80% of the cells as small embryoids of 3-5 cells each from suspensions having no embryoids at the start. It would thus be tempting to state that embryogenesis apparently is initiated in liquid suspension cultures, but as yet we have no proof. However, in the expectation of someone proving this type of embryogenesis, it is included in Table I as Type IV.

We have nearly completed the third year of a team effort at The Institute of Paper Chemistry, to determine the trigger mechanism of embryogenesis in conifers. In order to monitor biochemical feedback, we have developed a suspension assay based on the

percent green cells and the percent and size of embryoids. We have used this method during the past year to test many additives, and some have resulted in higher values than the controls. However, most of these treatments were added to the suspensions, and so we cannot say yet if they initiated embryoids or merely differentially enhanced their development in liquid.

Structure	Type I	Type II	Type III-A	Type III-B	Type IV
Embryoids	Explant	Callus	Callus	Callus	Liquid
Embryos	Explant	Callus	Liquid	Liquid	Liquid
Plantlets	Explant	Callus	Agar	Liquid	Liquid

TABLE I. TYPES OF EMBRYOGENESIS BASED ON SITES OF EMBRYOID INITIATION, EMBRYO DEVELOPMENT AND PLANTLET FORMATION

Note: The term embryoid conveys no commitment to further development. Rather it is a term of convenience designating a group of cells with nonrandom orientation that resembles the early stages of normal seed embryogenesis. However, the term embryo implies polarity and the ability to grow into a complete plantlet.

Experiments are already underway to try to determine the site of embryoid initiation in our suspensions, as well as trying to grow embryoids into embryos and plantlets. These problems will be discussed later. In this paper we report the types of embryoids produced in suspensions and our approach to quantifying the subjective observations made while monitoring the experiments microscopically.

## METHODS AND MATERIALS

Callus cultures were prepared from needles of young seedlings of Douglas-fir and loblolly pine, by sterilization and incubation methods reported earlier<sup>26,27</sup>. Green callus was isolated from the base of whole needles and subcultured monthly to fresh agar Medium 10<sup>27</sup> containing 5 mg/liter NOAA (naphthoxyacetic acid) and 0.1 mg/liter BAP (benzylaminopurine).

For the preparation of suspension inocula, soft callus was harvested three weeks after subculture, during the 4th-8th passage after isolation. About 50-100 cc of callus were macerated with forceps and divided among four 125-ml Erlenmeyer flasks, each containing 30 ml of liquid Medium 26-10 and covered with foil. Medium 26-10 is a simplified defined medium containing half the salts of Medium 10, with 400 mg/liter  $\text{NH}_4\text{NO}_3$ , 4000 mg/liter  $\text{KNO}_3$  and 35,000 mg/liter sucrose, and is sterilized by filtration.

Flasks containing the callus slurry were placed on a rotary shaker at 120 rpm in the light. After 2 hr, the slurry in each flask was diluted to 2-3 times the original volume and poured through a nylon screen having pores 1000  $\mu\text{m}$  in diameter. This removed about 20% of the callus as large masses of undissociated cells, but some large clusters of 50-100 cells went through the filter. All filtrate was combined and the volume in ml was increased to 10 times the total number of tubes in the experimental design. With constant swirling, the filtrate was then distributed among tubes 25 x 200 mm with metal closures, at 10 ml per tube and three tubes per treatment. Each tube received about 1 cc of loosely packed cells. After the filtrate was allowed to settle in the tubes for 20-30 min, all liquid was carefully decanted and replaced with the treatment solution. No more than 1% of the cells were lost during decanting.

The number of treatments ranged from 3-25, but averaged 7-10 per experiment. Feedback additives were incorporated into liquid Medium 26-10, filter sterilized and distributed at 10 ml per tube and three replications per treatment. An aliquot of treatment solution was poured into each of the first set of tubes, immediately following decanting of Medium 26-10 from the cell inoculum. This gave one set of tubes of treatment solutions inoculated with filtered and washed cells. These tubes were placed in a rollerdrum at 10-15 rpm under 4000-5000 lx of Cool-White and Plant-Grow fluorescent light, at 23-25°C. In early experiments all tubes were sampled daily or 2-3 times a week, but once a week has become standard. One sterile pipet (5 ml) was used to sample each tube for dark-field or phase-contrast examination with a Model I Zeiss Photomicroscope. Routine monitoring was recorded as three separate factors: (1) the percent of cells with green pigment, (2) the frequency of embryoids, and (3) the size of embryoids. Each of these were subjectively estimated during visual observations, with occasional checks for accuracy made by actual counts of cells in a few fields. On a scale of 1-10, each of these factors is shown in Table II.

## RESULTS

When we were developing Medium 26-10, several times we initiated small embryoids of 3-5 cells each, comprising 60-80% of the cells in suspension. However, the medium in these cultures was not replaced, and embryoids died within 1-2 weeks without enlargement. We also found occasional large embryoids of 100-200 cells each, but they had brown deposits in the cells and never grew into an embryo and plant. We now prolong the life of green cells in suspension by changing the medium 1-2 times a week. The addition of glucosamine will keep the suspensions green without changing, as will the replacement of inorganic with organic nitrogen, but these will not increase embryoid formation. We have been able to upgrade the general quality of our cultures simply by switching from 6-year old stem callus to young needle callus (a few months old) and changing the medium. However, some additives significantly

maintained green cells longer and promoted more embryoid formation than in controls. Some of these results will be given in an accompanying paper by Morris Johnson.

Value	Percent Green Cells	Percent Embryoids	Cells per Embryoid
1	0	0	0
2	1	1	5
3	5	5	10
4	10	10	15
5	25	20	20
6	50	30	30
7	75	40	50
8	90	50	70
9	95	75	100
10	100	100	+

TABLE II. DESCRIPTIVE VALUES FOR THE PERCENT GREEN CELLS, PERCENT EMBRYOIDS AND AVERAGE NUMBER OF CELLS PER EMBRYOID IN SUSPENSION CULTURES

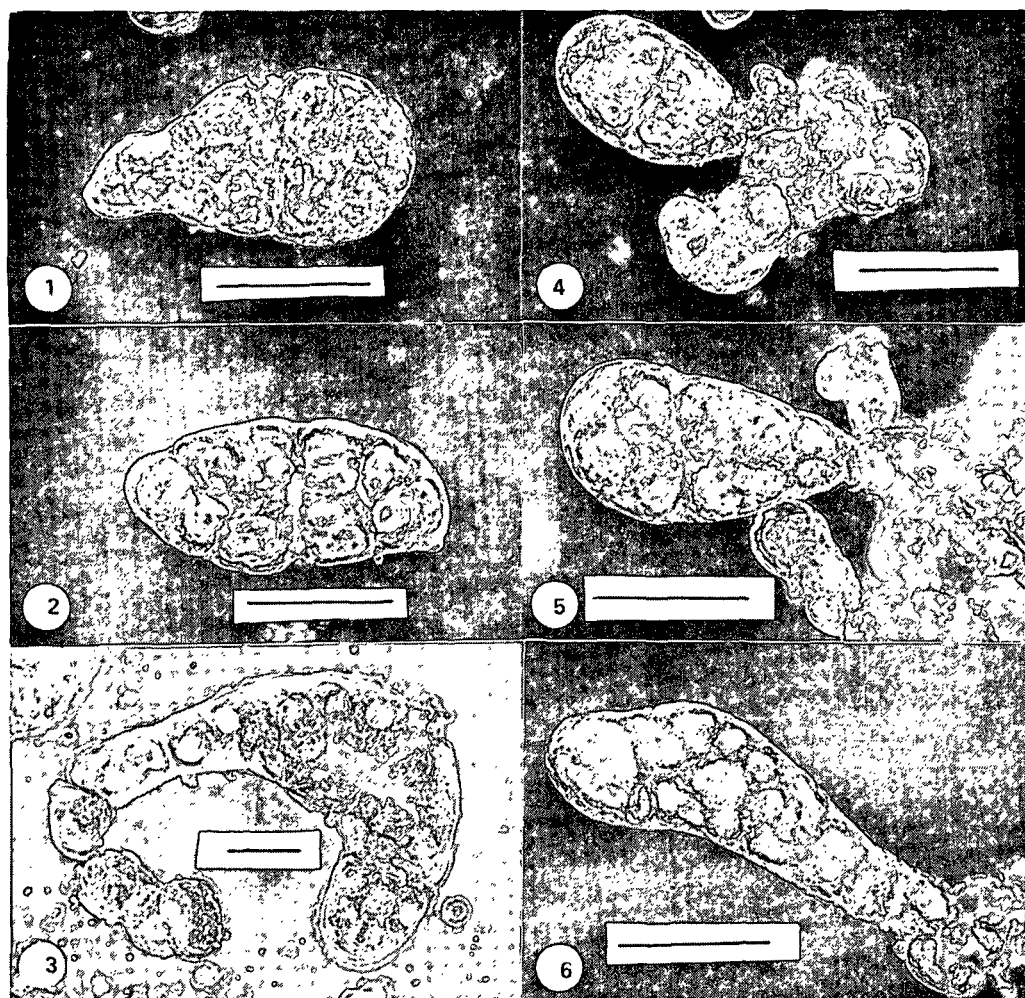
Our embryoids have been of two types: either with or without an attached cluster of cells. Those with the attached cluster probably arose from one cell in an existing small cluster of cells, and those without possibly formed from a single free cell. Rarely we have observed multiple embryoids from the same cell cluster, similar to early stages of multiple embryos reported in carrot<sup>15</sup>. However, most of our embryoids were single. Figures 1-3 show small, medium and large embryoids without attached cell clusters, and Fig. 4-6 show small and medium embryoids having attached clusters. All except Fig. 3 were produced in feedback experiments during the past six months. The large embryoid in Fig. 3 was produced in early experiments and had brown deposits.

We have also observed small and medium embryoids in suspensions of loblolly pine using similar techniques. However, we have had problems obtaining callus of loblolly pine, and only now are directing full attention to callus and suspension cultures of this species.

## DISADVANTAGES

We have not yet proven the primary assumption of our assay method: that these are embryoids, and that in an optimum environment they will develop into embryos and grow into plantlets. This we hope to demonstrate shortly, either by actually growing some into plants, or by finding ultrastructural differences between normal cell aggregates and embryoids that satisfy morphological criteria for embryogenic differentiation. Next we must determine the site of embryogenesis, whether in callus (Type III) or liquid (Type IV). If we have a Type III system, we may apply the feedback additives directly to callus, then transfer embryoids to suspension cultures for subsequent embryo development. If it proves to be a Type IV system, we can continue to use suspension cultures for testing additives.

Methods for the preparation of inocula and techniques used during monitoring must both be tightened for better control by reducing variability. We have done preliminary screening for cluster size using screens 500, 250, 125, and 75  $\mu\text{m}$ , and know that smaller cluster size is eventually necessary to study embryoid initiation. But first we must be able to



Figures 1-6. Embryoids from Douglas-fir suspension cultures. Figures 1-3. Embryoids without a basal cluster of cells representing small (Fig. 1), medium (Fig. 2) and large (Fig. 3) embryo cells. Figures 4-6. Embryoids with basal cluster of cells representing small (Fig. 4) and medium (Fig. 5-6) embryo cells. The scales all equal 100  $\mu$ m.

get nearly 100% cell dissociation in the callus slurry before filtration, possibly by using enzymes. The limits for each of the three factors in Table II must be fine-tuned to be amenable to quantification and statistical analyses. We should also look for ways to reduce the present subjectivity in monitoring, possibly by photometric methods.

#### ADVANTAGES

If we can demonstrate Type IV embryogenesis in our suspension cultures, not only can we continue to use this as a bioassay, but we may also be able to mass produce conifer trees from suspension cultures. This is a relatively simple assay, and since liquid medium must furnish all cell requirements, small changes in the medium should give large effects in growth and development. Results are also obtained faster in suspension cultures than in agar cultures, and suspension cultures occupy less space than callus cultures.

#### LITERATURE CITED

- Konar, R. and Natataja, K. *Phytomorphology* 15: 132-7, (1965).
- Kato, H. and Takeuchi, M. *Sci. Pap. Coll. Gen. Educ. Univ. Tokyo* 16:245-54, (1966).
- Haccius, B. and Lakshmanan, K. *Ost. Bot. Z.* 116: 145-58, (1969).
- Nitsch, C. Pollen culture - a new technique for mass production of haploid and homozygous plants. *In Haploids in Higher Plants* (K. Kasha, ed.), University of Guelph, 1974, pp. 123-35.
- Konar, R. and Oberoi, Y. *Phytomorphology* 15: 137-40, (1965).
- Bonga, J. Morphogenesis in cultures in balsam fir. *In Twenty-Seventh Annual Meeting of Tissue Culture Association*, June 7-10, 1976, Philadelphia, Paper No. 158.
- Reinert, J. *Naturwissenschaften* 45:244-5, (1958).
- Reinert, J. *Ber. Deut. Bot. Ges.* 71:15, (1958).
- Steward, F., Mapes, M., and Mears, K. *Am. J. Bot.* 45:705-8, (1958).
- Steward, F. *Am. J. Bot.* 45:709-13, (1958).
- Kato, H. and Takeuchi, M. *Plant Cell Physiol.* 4:243-5, (1963).
- Wetherell, D. and Halperin, W. *Nature* 200:1336-7, (1963).
- Halperin, W. and Wetherell, D. *Am. J. Bot.* 51: 274-83, (1964).
- Halperin, W. *Science* 146:408-10, (1964).
- Halperin, W. *Am. J. Bot.* 53:443-53, (1966).
- Vasil, I., Hildebrandt, A., and Riker, A.

- Science 146:76-7, (1964).
17. Wilmar, C. and Hellendoom, M. Nature 217: 369-70, (1968).
18. Sheridan, W. Planta 82:189-92, (1968).
19. Gamborg, O., Constabel, F., and Miller, R. Planta 95:355-8, (1970).
20. Durzan, D. and Steward, F. Bimon. Res. Notes, Can. Forest Serv. 24:30-1, (1968).
21. Durzan, D., Chafe, S., and Lopushanski, S. Planta 113:241-9, (1973).
22. Chafe, S. and Durzan, D. Planta 113:251-62, (1973).
23. Chalupa, V. and Durzan, D. Commun. Inst. Forestalis Chechoslov. 8:111-25, (1973).
24. Winton, L. Embryoids in suspension cultures of Douglas-fir. In Fourth North American Forest Biology Workshop, Syracuse, August 9-11, 1976.
25. Winton, L. and Huhtinen, O. Tissue culture of trees. In Modern Methods in Forest Genetics (J. Miksche, ed.), Springer-Verlag, Berlin, 1976, Chapter 12.
26. Winton, L. Forest Sci. 18:151-4, (1972).
27. Winton, L. and Verhagen, S. Can. J. Bot. (in press).

#### AUTHOR'S NOTE

We just received an excellent reprint from Bonga on the Application of Tissue Culture in Forestry, Chapter 5, in Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, edited by Reinert and Bajaj, Springer-Verlag, 1977. Of the 1113 references, at least 72 were of papers on embryogenesis in plants which we did not cite. We purposely omitted the many references to Citrus embryogenesis, but overlooked four papers on tree embryogenesis in coffee<sup>28</sup>, papaya<sup>29</sup>, rubber tree<sup>30</sup>, and holly<sup>31</sup>.

28. Staritsky, G. Acta Botan. Neerl. 19:509-14, (1970).
29. DeBruijne, E., DeLanghe, E., and van Rijck, R. Mededel. Fakult. Lanbouw Wetenschap. Gent 39: 637-45, (1974).
30. Paranjothy, K. Induced root and embryoid differentiation in Hevea tissue culture. In 3rd Int. Cong. Plant Tissue Cell Culture, Abstr. 67, Leicester, 1974.
31. Hu, C., and Sussex, I. Phytomorphology 21:103-7, (1971).



